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Title: Growth and Photosynthesis of Plants in Response to Environmental Stress

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Environmental stresses generally decrease photosynthetic rates and growth of plants, and alter biomass partitioning. Nutrient deficiency and drought cause root:shoot ratios to increase, whereas the air pollutant ozone (O_3) causes an opposite shift in carbon allocation. Plants in nature usually grow under suboptimal conditions; therefore plants were raised with O_3 combined with other stresses to analyze the mechanisms whereby multiple stresses influence gas exchange and growth.

Physiological and growth responses to stress were determined for radish (*Raphanus sativus*), soybean (*Glycine max*) willow (*Salix nigra*), alder (*Alnus serrulata*) and aspen (*Populus tremuloides*) in laboratory and field trials. These species were chosen to represent a range of growth forms and rhizosphere associations.

In willow, high-nutrient status plants had more visible injury, but a smaller decline in leaf area with O_3 than did low-nutrient plants. Nutrient deficiency prevented the root:shoot ratio shifts expected in response to O_3. The relationship between photosynthesis and conductance in alder was altered by O_3, and recovery from O_3 was impaired in plants lacking root nodules formed by the N_2-fixing symbiont *Frankia*. Ultrastructure of host plant cells in alder root nodules was disrupted by O_3, suggesting that this air pollutant can affect the ability of plants to acquire nutrients via symbioses.

Biomass and root:shoot ratios decreased with O_3 in radish and soybean. Shifts in stable carbon isotope ratios were caused by O_3,

and this technique was used to integrate the effects of O_3 on gas exchange over time. In aspen, O_3 enhanced photosynthesis and foliar areas in young leaves of well-watered aspen, partially compensating for declines in older leaves. This effect was more pronounced in plants raised at a high nitrogen level than in N-deficient plants. Carboxylation efficiency decreased in older, but increased in younger leaves with O_3. Prior exposure to drought reduced effects of O_3 on photosynthesis and leaf area. Collectively these studies show that addition of other environmental stresses can alter plant response to O_3.

Growth and Photosynthesis of Plants in Response to Environmental Stress

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GROWTH AND PHOTOSYNTHESIS OF PLANTS IN RESPONSE TO ENVIRONMENTAL STRESS

I. INTRODUCTION

Analysis of plant-environment interactions

Environmental stresses are those factors that affect the physiology or metabolic processes of plants so as to limit growth. Plants in natural environments, managed forests, and agricultural fields commonly encounter more than one stress during their life cycles. Nutrient deficiency, lack or excess of water, and extremes of temperature and light intensity are common natural stresses which may occur singly or in combination. Stresses are also imposed by human modification of the environment. Since environmental stresses have long been known to limit productivity of trees and crops, much research in plant sciences has focused on the topic of plant response to these factors. This research includes analysis of individual plants and plant communities, and studies in plant physiology (Jones, 1983, Fitter & Hay, 1987).

In spite of all the studies conducted on this topic, much remains to be done. Many studies to date focused only on analysis of plant responses to single stresses, such as drought, nutrient deficiency, or air pollution. Such stresses rarely occur singly; plants grow in environments where they encounter combinations of limiting factors. For example, drought may commonly coincide with temperatures above the optimal for plant physiology and growth.

The need for analysis of plant responses to stress combinations poses difficult challenges: there are numerous possible stress combinations and several hundred thousand plant species to consider. Even with these challenges, the need for scientific advancement is compelling. The environment of the earth, including components of its physical and chemical climate, is changing now as it has through the millenia. In addition, human activity is further

modifying global climate in ways that are not entirely understood or predictable. Given the uncertainties of the rate, extent, and magnitude of environmental change, those concerned about native and managed plant populations must develop the tools to predict plant responses to environmental stresses based upon general principles that govern plant-environment relations.

One important concept that has emerged is that plants can compensate physiologically to natural stresses so that acquisition of limiting resources and growth are maximized under the prevailing conditions. Compensation has been demonstrated with nutrient deficiency (Bloom, Chapin & Mooney, 1985) and drought stress. For example, plants generally compensate to stresses from below-ground (nutrient or water deficiencies) by shifting resource allocation patterns to favor root growth over shoot growth (Marschner, 1986; Sharp & Davies, 1989). In contrast, plants subjected to above-ground stresses such as low light intensity compensate by altering allocation to favor shoot growth over root growth. Thus growth of the part of the plant responsible for acquiring the limiting resource is maintained at the expense of other plant organs. In addition to shifting root:shoot ratios, plants may compensate to stresses by other mechanisms involving physiological and growth processes that are related to changing the patterns of resource acquisition, assimilation and allocation. The factors driving these shifts may be mere proximity to the limiting resource or a more organized stress response mediated by hormonal control (Chapin, 1980).

Plants also shift root:shoot ratios to compensate to air pollution stress (Darrall, 1989). As these shifts are opposite in direction to the shifts expected with the common natural stresses of nutrient and water deficiencies, the effect of air pollution combined with these natural stresses is difficult to predict.

Air pollution has the potential to alter plant responses to other stresses. Air pollution is a problem that is regional and international in scope, and occurs where plants are exposed to natural stresses. Plant physiology, growth, and yield are known to

be altered by several types of air pollutants including O_3, SO_2, NO_x, and acid deposition (Guderian, 1985; Winner, Mooney & Goldstein, 1985; Reuss & Johnson, 1986; Schulte-Hostede, 1988; National Research Council, 1989; Olson & LeFohn, 1989; Schulze, Lange & Oren, 1989; Smith, 1990).

Ozone (O_3) is the gaseous air pollutant that poses the greatest threat to plants. This threat stems from the high degree of O_3 phytotoxicity and its widespread distribution across much of North America and other continents. It is formed from reactions involving hydrocarbons and nitrogen oxides (NO_x) emitted from fossil fuel combustion in the presence of sunlight and O_2. O_3 and its precursors can be transported great distances so that many plants in agricultural fields, managed forests and even in relatively pristine undisturbed areas are being exposed to this pollutant.

Research approach and questions

The information outlined above raises several questions about plant response to combinations of anthropogenic and natural stresses. In this thesis project, I worked with willow, alder and aspen which are perennial, woody, deciduous species. I also worked with radishes and soybeans which are annual crop species. My choice of species was governed by the types of experiments needed to address each individual question.

I also chose to conduct experiments with both natural stresses and gaseous air pollutants. The natural stresses I selected for study were nutrient deficiency and drought. These stresses are common and are of great economic and ecological importance. I chose O_3 as the anthropogenic stress to study because of its wide distribution, high level of phytotoxicity, and the volume of information available on its effects. The experiments consisted of raising the plants in either laboratory or field chambers where factors could be monitored and controlled. Plant responses to environmental regimes were determined by physiological measurements and by harvests to characterize patterns of growth. These experiments were designed to answer four interrelated questions:

1). How does nutrient level affect O_3-caused visible injury and growth? Visible injury has often been studied as a response to pollutants, but the link between foliar injury and decreased growth is not clear. I explored the relationship between foliar injury and growth with a relatively simple system. Willow plants were raised from cuttings in a greenhouse where nutrient level was controlled by addition of fertilizer. O_3 was applied in laboratory fumigation chambers. The value of these experiments is that in this system, no rhizosphere symbionts capable of affecting the nutritional status of the plants were included. Thus this was a test of O_3 - nutrient stress interaction in a straightforward case.

2). Can changes in shoot physiology with O_3 affect rhizosphere symbioses and therefore the ability of plants to acquire nutrients? Fumigation with O_3 can be detrimental to establishment and functioning of mycorrhizae and *Rhizobium*. The goals of this study were to link O_3-caused changes in photosynthesis with changes in rhizospere symbiosis, and to determine whether O_3 can affect the ability of plants to acquire nutrients via symbioses. Alder was the test species and it was grown in a greenhouse and laboratory chambers with and without *Frankia*, a N_2-fixing symbiont.

3). Are photosynthesis measurements taken during a brief window of time representative of plant physiology throughout a prolonged exposure to stress? Measurements of photosynthesis involve taking samples of foliar gas exchange during less than a minute for each individual leaf. Photosynthetic rate may in some conditions be highly variable throughout a day or season, or in the presence of a diurnally changing stress such as O_3. It would be advantageous to correlate these transient measurements with some other technique that could serve as an integrator of changes in plant metabolism. Analysis of stable carbon isotope ratios of plant tissue provides such an integrator. Controlled laboratory experiments were conducted with soybean and radish exposed to O_3 and growth, photosynthesis, and stable carbon isotope ratios were all measured.

4). How are photosynthesis and leaf area of whole plants affected by O_3 in nitrogen deficient plants and in plants recovering from drought? Does the response vary with leaf age, and how do plants compensate to these stresses? In these field experiments, three stresses were examined in aspen, a woody deciduous perennial. In most studies of plant physiological response to stress, one or a few leaves per plant are sampled. As response to environmental factors can change with age, this approach cannot provide a complete understanding of how the whole plant is responding to the stress. In this study, photosynthesis and leaf area of entire plant canopies were measured in order to understand the response of the whole plants to stress.

Taken together, these questions focus on an analysis of plant responses to below ground stress, i.e. nutrient deficiency and drought, in combination with an atmospheric stress, O_3. Although not entirely definitive, these experiments show that plant responses to below ground stress affect plant responses to O_3 and that foliar injury is not related to changes in growth. Further, this analysis shows the difficulty of conducting multifactorial designs but shows the potential for laboratory and field experiments that are carefully designed to analyze plant responses to stress combinations.

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II. NUTRIENT EFFECTS ON RESPONSES OF WILLOW AND ALDER TO OZONE

Greitner, C. S. & Winner, W. E. (1989b). Nutrient effects on responses of willow and alder to ozone. In: *Transactions: Symposium on the Effects of Air Pollution on Western Forests* (Ed. by R. K. Olson & A. S. Lefohn), pp. 493-511. Air & Waste Management Association, Pittsburgh, PA.

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INTRODUCTION

Nutrient status is known to influence plant response to air pollution. Unfortunately, mechanisms of this interaction are not well understood, with no general trends apparent from the reports in the literature. It is important to understand any nutrient-air pollutant interaction, both for considering fertilizer applications for crops in highly polluted areas and for evaluating pollutant effects on plants in forests and other natural settings. In regions where acid deposition occurs, nutrient availability may change, thereby altering the response of crops and native vegetation to gaseous pollutants such as O_3 and SO_2.

To make matters more complex, most crops and native plants grow with rhizosphere symbionts including mycorrhizae and nitrogen fixing bacteria. These organisms play important roles in increasing availability of nutrients such as N, P, and K to plants. Air pollutants may influence the symbiosis thereby altering the capacity for plants to acquire nutrients and to respond to air pollution.

One goal of this paper is to synthesize the current understanding in this area by examining reports in the literature in which plants were raised under various nutrient regimes and exposed to O_3. We have not considered studies with SO_2 and acid deposition due to the complication that these air pollutants include elements which are nutrients in themselves.

Another goal is to probe nutrient-air pollutant interactions with a simple plant system without rhizosphere symbionts. We conducted a laboratory study with willow (*Salix nigra* Marsh.) to attempt to determine 1) if nutrient status affected this species' sensitivity to O_3 and 2) if visible injury was associated with growth reduction. Willow was chosen because stem cuttings readily produce adventitious roots, making it possible to quickly obtain many genetically-alike individuals.

A final goal of this paper is to relate O_3-nutrient interactions to the capacity for rhizosphere symbionts to influence plant response to O_3. This involved exposing alder (*Alnus serrulata* Aiton. Willdenow) with and without root nodules formed by the nitrogen-fixing actinomycete *Frankia* to filtered air or O_3. Thus high and low nitrogen treatments were provided by the presence and absence respectively of the rhizosphere symbiont. Gas exchange parameters were measured. In addition, stable carbon isotope ratios were analyzed for both nodulated and unnodulated plants.

Current understanding

Nutrients are seldom at optimal levels for plants. In general, during vegetative growth phases, plants compensate for nutrient oversupply and undersupply in order to 1) maximize growth under prevailing conditions and 2) maximize acquisition of limiting resources (Bloom, Chapin & Mooney, 1985). Nutrient levels are typically below optimal; plants compensate by increasing root:shoot ratios (Marschner, 1986). Interestingly, air pollutants are known to cause decreases in root:shoot ratios (Reinert, Shriner & Rawlings, 1982; Tingey, Heck & Reinert, 1971; Walmsley, Ashmore & Bell, 1980). Thus plants exposed to both insufficient nutrient supply and air pollution are coping with stresses that would independently force resource partitioning in opposite directions. The "ideal" of optimal nutrient level and clean air results in a carbon allocation pattern balanced between root and shoot (Fig. II.1a). Nutrient deficiency in clean air leads to a shift in allocation to enhance root growth at the expense of shoot growth (Fig. II.1b). When nutrients are at optimal levels but O_3 is present, the allocation pattern is shifted in the opposite direction and shoot allocation is dominant (Fig. II.1c). The overall size of the plant may also be reduced. The effect of combining nutrient deficiency and O_3 exposure is largely unknown (Fig. II.1d). Of principal concern then is the relative strength of these interacting stresses as they drive plant morphology in opposite directions.

Nitrogen is generally thought to be widely limiting for plant

production in both agricultural and forested systems. However, excess N has the potential to damage some forests because balances in growth between roots and shoots may be disrupted by direct fertilization of the canopy (McLaughlin, 1985). N is positively correlated with metabolic capacity: plants raised at high N have higher photosynthetic capacities and stomatal conductances than plants raised at low N (Wong, Cowan & Farquhar, 1979). These metabolic parameters are directly related to air pollution sensitivity; i.e. high conductance results in high pollutant absorption rates Winner & Mooney, 1980a, b; Reich & Amundson, 1985).

N status is also known to alter plant sensitivity to air pollution, but results vary from study to study. Tobacco plants grown at optimal N level showed more foliar injury than plants grown at either deficiency or luxury levels in one report (Leone, Brennan & Daines, 1966), but in another case injury was enhanced by both deficiency and excess of N (MacDowall, 1965). N had no interaction with O_3 in growth of fescue and ladino clover (Montes, Blum & Heagle, 1982). High N concentrations made *Lemna minor* more susceptible to loss of chlorophyll induced by O_3 (Craker, 1971).

K is involved in stomatal control (Marschner, 1986) and therefore may influence the amount of O_3 absorbed by leaves. Plants raised under K deficiency might be unable to open stomata as widely as plants whose K supply was not limiting. K-deficient plants might therefore absorb less O_3 and express less injury. Studies with silver maple (Noland & Kozlowski, 1979) and tomato (Leone, 1976) gave results compatible with this scenario. In contrast, soybean and pinto bean grown at low K showed more foliar injury than at high K (Dunning, Heck & Tingey, 1974).

Increased levels of P were associated with increased visible injury in tomato (Leone & Brennan, 1970). High levels of S protected against both visible injury and loss of chlorophyll induced by O_3 in beans (Adedipe, Hofstra & Ormrod, 1972). Hybrid poplars grown at half the recommended rate of NPK fertilizer had more visible injury than unfertilized plants and those raised using the recommended fertilization rate (Harkov & Brennan, 1980).

Relative proportions of individual nutrients appear to be important in injury expression (Craker, 1971).

Studies on nutrient impact on plant susceptibility to O_3 have been conducted with different species, nutrient levels, and O_3 doses. Few studies have involved more than one or two species or nutrient elements; thus drawing general conclusions about nutrient-pollutant interactions is difficult. Experiments involving several species and relating visible injury to changes in growth and physiology are needed.

MATERIALS AND METHODS

Willow Study

Branches were collected from a single *Salix nigra* shrub in the Jefferson National Forest, Montgomery County, Virginia in October, 1985. Cuttings were placed in trays of Promix (Premier Brands, Inc., New Rochelle, New York) under mist in a filtered-air greenhouse with a 16 hour light photoperiod. After 4 weeks, cuttings with roots and leaves were removed from the mist and transplanted to individual 0.7 liter pots containing Promix. About a month later, roots of the cuttings were washed free of potting mix and transplanted to pots containing an inorganic mixture of 1 volume clay to 3 volumes vermiculite. Plants were fertilized with 1 (low nutrient plants), 2 (medium nutrient), or 3 (high nutrient) grams slow-release Osmocote 14-14-14 (NPK) fertilizer (Sierra Chemical Company, Milpitas, CA) per pot.

For each of the three fertilizer levels, 12 plants were randomly selected to be exposed to O_3-free charcoal-filtered air (controls), 12 to be exposed to 0.06 ppm O_3 (low O_3) and 12 to be exposed to 0.12 ppm O_3 (high O_3). O_3 was generated from pure O_2 by electrical discharge. Fumigations began about 4 weeks after fertilization and were conducted in continuously stirred tank reactors (CSTRs) equipped with 1000 W metal halide lamps. Plants were fumigated an average of 4 hours per day for 18 days of a 27 day

period. Six chambers were used for the study (two control, two low O_3 and two high O_3) and six plants grown at each nutrient level were placed in each chamber. Plants were returned to the greenhouse after exposures. The day after each fumigation, foliage was inspected for visible injury, with percent leaf area necrotic estimated to the nearest 5% for each plant. When only a trace of injury (necrosis of less than 5% of the total leaf area) occurred, the plant was given a rating of 1%.

During the 11th exposure, one chamber of control plants was lost due to equipment malfunction. Therefore, the number of control plants per nutrient treatment in the final harvest was six, half that of the low and high O_3 exposures. Foliar injury had not been observed on any of the plants in the low or high O_3 chambers after 11 exposures; therefore the mean concentrations were doubled to an average of 0.12 ppm in the low and 0.24 ppm in the high O_3 chambers in order to determine the sensitivity of *S. nigra* to O_3. There were seven exposures to the increased concentrations, and the final harvest took place 2 - 3 days after the last fumigation. Roots were washed to remove potting mix, and the plants were separated into roots, stems, and leaves. Leaf areas were measured with a Hayashi Denko AAM-5 area meter. Samples were dried in a forced-air oven for 48 hr and weighed to 0.01 mg. Means and standard errors of the means were calculated.

Alder - *Frankia* Study

Alnus serrulata seeds were collected from a single clump of stems in the Jefferson National Forest in November 1985. Seeds were surface sterilized in bleach (5.25% sodium hypochlorite) for 5 - 10 minutes, rinsed in deionized H_2O, and soaked in deionized H_2O for 3 days. Seeds were drained, placed in a plastic bag, and stratified for 41 days in a cold room (about 4 °C) followed by 3 days in a freezer (about -10 °C). Seeds were sown in pots containing a 1:3 v:v mixture of clay and vermiculite in the filtered air greenhouse with a 16 hour light photoperiod. Seedlings emerged after several weeks and were watered daily. About 3 months after sowing,

seedlings were transplanted to individual 0.7 liter pots containing 0.5 g Osmocote 14-14-14 fertilizer.

Five and a half months after planting, 17 seedlings were selected to be inoculated with *Frankia*. Root nodules collected in the field were washed in deionized H_2O, blotted dry, weighed, surface sterilized in bleach for 15 minutes, and rinsed in deionized H_2O. The nodules were ground with a mortar and pestle, with sufficient deionized H_2O to make a 0.07 g ml^{-1} suspension. Two ml of the suspension were injected into the potting mix in several locations near the roots in each pot. Seventeen other seedlings received deionized H_2O. By approximately 2.5 months after inoculation, new leaves of the successfully nodulated plants were observed to be larger and greener than those of unnodulated plants.

Fumigations began in the CSTRs 8.5 months after seeds were sown. Exposures to either 0.12 ppm O_3 or O_3-free air were administered for 27 out of 29 days for periods ranging from 4 to 11 hours per day. Fumigated plants were exposed to O_3 for a total of approximately 164 hours. Plants were returned to the lighted greenhouse bench following each fumigation to maintain the 16 hour daylight regime. Eight chambers were used for the study (four O_3 and four O_3-free control) and one pot each of nodulated and unnodulated alder was selected for treatment in each chamber.

A LI-COR LI-6200 photosynthesis system was used for gas exchange measurements inside the fumigation chambers, which had doors equipped with gloves for cuvette manipulation. Measurements were made 24 and 29 days into the fumigation period, with the two days' data combined and analyzed together. A young, fully expanded leaf of each control and O_3-treated seedling was measured when plants were in the chambers in filtered air between fumigations (a total of four readings per treatment taken on each of the two measurement days). Leaves of O_3-treated plants were measured again at least one hour after exposure to O_3 began. Photosynthesis, stomatal conductance, and internal CO_2 concentration were calculated by the system software. Ambient CO_2 concentrations in the cuvette were less than 387 ppm immediately before measurements were taken.

Means for the eight readings for each treatment were calculated, and significant differences were based on the standard error of the mean. Linear regressions were calculated for plots of photosynthesis versus conductance for each treatment. Total foliage was collected from each plant, dried at 60°C for 48 hours and ground to pass through a #40 mesh. Samples were analyzed for stable carbon isotope ratios by Dr. James Ehleringer, University of Utah (Greitner & Winner, 1988).

RESULTS

Simple Nutrient Supply System: Willow Study

Foliar Injury

No injury was visible after 11 exposures (45.5 hours) at the original mean O_3 concentrations of 0.06 and 0.12 ppm. After one 4 hour exposure to the doubled concentrations, injury appeared on many plants exposed to the high O_3 treatment. O_3-induced foliar injury first appeared as dark purple areas on the laminas of middle-aged leaves in particular, and older and younger leaves to a lesser extent. Injured areas became necrotic, and when injury covered a large area, leaf abscission was common. Area of abscised leaves was not included in the evaluation; therefore the percent leaf area injured scores of highly injured plants sometimes decreased with increasing number of fumigations as an artifact of the scoring procedure. Thus the total leaf area lost as a result of exposure to high O_3 is under-represented by the visible injury scores for the most severely injured plants in the later fumigations. In the high O_3 treatment, percent leaf area injured of high and medium nutrient plants was greater than that of low nutrient plants (Fig. II.2). Number of plants showing injury after each fumigation increased with fertilizer level until all plants exposed to high O_3 expressed some foliar injury (Fig. II.3). Thus nutrients also influenced the percentage of plants showing injury (Fig. II.3).

Similar patterns of foliar injury development were observed

for the low O_3 treatment. Percent leaf area injured was low (means of 1 to 2%, with no plant showing more than 5% leaf area necrotic) at all nutrient levels. Injury appeared earliest on the high nutrient plants, and the number of plants expressing visible injury increased with increasing fertilizer level at low O_3 (Fig. II.4).

Surprisingly, foliage was not injured when plants were exposed initially to O_3 at 0.12 ppm for 11 fumigations, yet injury appeared on the plants previously exposed to 0.06 ppm after two fumigations at 0.12 ppm. Injury was observed on at least half of the low O_3 plants of each nutrient treatment after seven fumigations at 0.12 ppm. Since there were leaves of all ages on the plants throughout the experiment, it is unlikely that this effect was simply a matter of leaf age or timing of the exposures in the life cycle of the plants. Rather, the previous exposure to 0.06 ppm seems to have predisposed the low O_3 plants to injury by 0.12 ppm. Possibly, initial exposure to 0.12 ppm O_3 induced rapid partial stomatal closure in plants that had never been exposed to O_3, but exposure to 0.06 ppm O_3 did not. The stomata might have acclimated to O_3 at a low concentration and then failed to close when subsequently exposed to 0.12 ppm.

Analysis of Willow Growth

Willow cuttings responded to increasing nutrient levels with increases in leaf area and in dry weights of leaves, stems, and roots (Table II.1). Leaf weight and area increased with nutrient level within all air pollution treatments. Root and stem weights always were greater at medium than at low nutrient level. High nutrient plants sometimes, but not in all cases, had greater stem and root weights than did medium nutrient plants.

Root:leaf ratios were calculated to detect changes in resource allocation patterns (Table II.2). Root:leaf ratios were expected to decrease with increasing fertilizer application rate. This tended to be true for low and high O_3 plants, but the trend was not as clear for control plants, perhaps due to the smaller number of controls. High nutrient control plants did however have a lower

root:leaf ratio than either the low or medium nutrient controls.

Mean leaf area decreased with increasing O_3 within all nutrient treatments (Table II.1). In all cases, there was a trend for mean leaf weight to decrease with increasing O_3 (Table II.1). Stem weight was unaffected by O_3. The only large effect of O_3 on root weight was a 34% reduction for medium nutrient high O_3 plants.

High O_3 caused a mean decrease in leaf area of 28% (Table II.1) and a similar decline in leaf weight (25%, Table II.1) for low nutrient willow cuttings. Mean percent foliar injury (maximum recorded was 10%) was not large enough to account entirely for the loss in leaf area or weight. Root:leaf ratio increased, showing that resource allocation had shifted to favor growth of roots at the expense of leaves (Table II.2). The declines in leaf area and weight relative to controls were apparently a result of both losses from necrosis and changes in allocation patterns. At low O_3, low nutrient plants had only a trace of injury, yet leaf area and weight declined (by 19% and 15% respectively, Table II.1). Again, root:leaf ratio tended to increase compared with controls (Table II.2). Resource allocation shifts favoring root growth seemed to be solely responsible for the decline in leaf growth for low nutrient, low O_3 willows. These results for low nutrient plants indicate that nutrient deficiency was stronger than O_3 in influencing allocation.

For medium nutrient plants exposed to high O_3, there was a decline in leaf weight (25%, Table II.1) which was similar in value to the percent area necrotic (more than 16%) and percent decline in leaf area (19%) (Table II.1). Root weight decreased 34% relative to controls. Root:leaf ratios decreased with increasing O_3 level for medium nutrient plants, as expected (Table II.2). Carbon allocation shifted to favor leaf growth at the expense of root growth and may have helped compensate for the loss of foliage due to necrosis. Apparently medium nutrient plants were not nutrient deficient and responded to the atmospheric stress by shifting allocation to the foliage.

High nutrient willow cuttings exposed to high O_3 had a mean decrease in leaf area of at least 19% and in leaf weight of 12%

(Table II.1) relative to the corresponding controls. Unexpectedly, root:leaf ratios tended to increase with increasing O_3 for high nutrient plants (Table II.2). This increase was a consequence of O_3-caused reduction in leaf weight, along with a tendency for root weight to increase with O_3. The explanation for this trend is not clear. The high nutrient level did not appear to have induced toxicity symptoms.

Complex Nutrient Supply System: Alder-*Frankia* study

O_3-caused injury symptoms were not observed on any leaves. Unnodulated plants always had lower photosynthesis (Fig. II.5) and stomatal conductance (Fig. II.6) and higher CO_2 internal values (Fig. II.7) than did nodulated alders, presumably as a consequence of N deficiency. Mean photosynthetic rate of nodulated plants was not decreased by O_3 (Fig. II.5). Plants without *Frankia* had about a 40% decrease in mean photosynthesis in response to O_3 (Fig. II.5), which although not statistically significant, could be of biological importance. In addition, conductance of nodulated and unnodulated plants tended to increase with O_3 treatment, both during fumigation and in filtered air (Fig. II.6). Normally, factors that increase conductance make CO_2 more available to mesophyll cells and photosynthesis also increases.

Wong, Cowan & Farquhar (1979) observed that photosynthetic capacity and conductance were closely and positively correlated in *Zea mays*. Lange, Beyschlag, & Tenhunen (1987) cited several studies showing a linear relationship between CO_2 assimilation and conductance. To test whether such a linear relationship existed for alder, photosynthesis was plotted against conductance for leaves of control plants and plants that had received O_3.

Photosynthesis and conductance were positively correlated for control seedlings (Table II.3). No significant positive correlation was found for leaves of nodulated or unnodulated O_3-fumigated plants measured during exposures (Table II.3), because leaves which had high conductance had lower photosynthesis values than expected.

Gas exchange rates of leaves of O_3-treated nodulated and

unnodulated seedlings were also measured in filtered air to determine whether O_3 effects lasted beyond the fumigation period. Photosynthesis and conductance were correlated (Table II.3) for leaves of nodulated plants. Thus the relationship between photosynthesis and conductance for O_3-treated leaves of nodulated plants recovered in clean air. However, photosynthesis and conductance were only weakly correlated for O_3-treated leaves of unnodulated plants measured in filtered air (Table II.3). N deficiency apparently reduced the capacity for these leaves to return completely to the expected relationship between photosynthesis and conductance.

Sensitivity to O_3 was associated with high conductance for leaves of both nodulated and unnodulated plants, presumably because leaves with high conductance absorb more air pollutant. In addition, leaves with high values of conductance or photosynthesis are more metabolically active, and high rates of metabolism may confer sensitivity to air pollutants (Winner & Mooney, 1980a). If the observation that the leaves with highest metabolic rates are also most sensitive physiologically to O_3 is generally true, then it would appear that those leaves contributing most towards productivity are most vulnerable to O_3.

Stable carbon isotope ratios, expressed as delta ^{13}C values, were measured for leaves of control nodulated and unnodulated alders (Fig. II.8). Leaves of plants lacking *Frankia* had significantly more negative delta ^{13}C values than plants with the symbiont. These values, which are an integrated expression of the physiological status of the plants throughout their growth, were in agreement with the instantaneous gas exchange measurements. The CO_2 internal value (Fig. I.7) was higher for unnodulated plants, indicating that ribulose bisphosphate carboxylase was able to discriminate against ^{13}C to a greater extent in unnodulated than in nodulated plants. This resulted in the more negative delta ^{13}C values for the N-deficient plants. N deficiency evidently reduced photosynthesis more than conductance, since CO_2 internal values were higher for unnodulated alders. Determining delta ^{13}C values for control and O_3-

treated plants raised at various nutrient levels would provide more information on the effect of nutrition on O_3 response.

DISCUSSION

The majority of reports in the literature on O_3 - nutrient interactions have focused on visible injury. The results are mixed: often plants raised at optimal nutrient levels showed enhanced O_3-induced foliar injury, but there are many exceptions. Few studies have examined nutrient influence on O_3-caused effects on growth or physiology; these areas require more attention. One important feature of air pollution and nutrient stress interactions which has long been overlooked involves biomass partitioning between roots and shoots. Since air pollution stress results in partitioning favoring shoots and nutrient deficiency results in partitioning favoring roots, these two stresses together place the fate of photosynthate in question. No concepts have emerged enabling the prediction of carbon partitioning for plants faced with these two types of stress.

For willow, increased nutrient status resulted in increased visible injury. However, nutrient-deficient plants were more sensitive to O_3-induced reductions in leaf area than were plants raised at higher nutrient levels. Thus foliar injury, which is recognized as a poor predictor of growth responses to gaseous pollutants, is quite misleading in this study. More specifically, high foliar injury occurred for plants which on the basis of growth analysis were more resistant to O_3.

Nutrient level and O_3 influenced carbon allocation patterns. The willow study showed that, in general, nutrient deficiency can be so severe as to prevent the biomass partitioning expected in response to O_3. For low nutrient willows the effect of poor nutrition was stronger than the effect of O_3 in determining carbon allocation, because leaf area and weight decreased relative to controls but root weight did not. At higher levels of nutrient availability, nutrients and O_3 both seemed to influence

partitioning. In medium nutrient plants, carbon allocation shifted to favor leaf growth at the expense of root growth, in agreement with the general finding that air pollutants affect root growth more than shoot growth. Whether O_3 stress can ever be severe enough to override nutrient deficiency and dominate partitioning has yet to be determined.

The alder - *Frankia* system is more complex than the willow system in several ways. For example, photosynthate in the willow system is partitioned only between roots and shoots. In the alder system, photosynthate can go to roots, shoots, and symbiont. Carbon allocated to the symbiont can be viewed as either lost from the plant or as a third allocation pool that is well integrated with the tree. The rules for stress effects on carbon allocation are understood for roots and shoots, but the rules governing allocation to *Frankia* are unknown.

In addition, *Frankia* can play a direct role in uncoupling alder from the constraints of soil N. Thus interpreting the relative nutrient status of alder in stress experiments is difficult. More specifically, we could manipulate N content in potting media for willow and be certain of low, medium and high N treatments. For alder, low soil N enhances the *Frankia* symbiosis, and activity of the symbiont can contribute substantial amounts of N to a low fertilizer treatment. Thus obtaining alder at high and low N levels is not simple. Our study exploits the fact that photosynthetic capacity is an excellent assay for foliar N because the two are correlated.

The results with alder can be viewed in context of those for willow. Some of the leaves from unnodulated alders had such poor nutritional status that O_3 had no effect on gas exchange; the same was true for nodulated alder. Only leaves with high conductance showed an O_3 response, for plants in both nutrient treatments. Thus as with willow, the severity of one stress (low nutrients) can be so profound that the effects of a second, lesser stress (O_3) are overwhelmed. These experiments were conducted with species native to the eastern U. S.; however species of *Alnus* and *Salix* occur

throughout the Pacific Northwest. The effects of nutrient and symbiont status on plant response to O_3 reported here are expected to be similar for western members of these two genera. Changes in photosynthesis and carbon allocation patterns observed in these studies may be general plant responses that are applicable to a wide range of woody species.

However, the possibility exists that for some species or conditions, altering the fertilizer level will have no effect on plant response to O_3. That is, O_3 may cause the same percent reduction in growth for plants of low and high nutrient status. This might be expected in species in which nutrient deficient individuals have the same photosynthetic rate and same percent foliar nitrogen, but smaller overall biomass, as for high nutrient plants. Additional work with other species will reveal whether the results reported here for willow and alder are widely applicable or restricted to these experimental conditions.

ACKNOWLEDGEMENTS

This research was supported by U. S. Department of Energy Grant DE-FG05-85ER60312 to W. E. W. We thank James Ehleringer for the isotope analysis and helpful comments.

Table II.1. Dry weights (gm) and leaf areas (cm^2) of *Salix nigra* cuttings grown at three nutrient levels and exposed to filtered air or low (0.06 ppm increased to 0.12 ppm) or high (0.12 ppm increased to 0.24 ppm) O_3. Values are means (n=6 for controls, n=12 for O_3-treated cuttings) ± standard errors.

Treatment	Dry Weight Leaves (gm)	Dry Weight Stems (gm)	Dry Weight Roots (gm)	Leaf Areas (cm^2)
Control, Low Nutrient	2.23 ± 0.22	1.78 ± 0.27	2.85 ± 0.67	620 ± 53
Control, Medium Nutrient	3.23 ± 0.26	2.94 ± 0.27	4.98 ± 0.71	915 ± 54
Control, High Nutrient	3.76 ± 0.24	3.35 ± 0.23	3.68 ± 0.55	1088 ± 67
Low O_3, Low Nutrient	1.89 ± 0.14	1.77 ± 0.17	3.12 ± 0.39	504 ± 37
Low O_3, Medium Nutrient	3.14 ± 0.22	2.91 ± 0.29	4.50 ± 0.66	895 ± 53
Low O_3, High Nutrient	3.65 ± 0.25	3.25 ± 0.29	4.16 ± 0.63	1037 ± 60
High O_3, Low Nutrient	1.70 ± 0.15	1.73 ± 0.17	2.88 ± 0.41	444 ± 36
High O_3, Medium Nutrient	2.78 ± 0.15	2.64 ± 0.13	3.27 ± 0.33	737 ± 39
High O_3, High Nutrient	3.30 ± 0.24	3.22 ± 0.29	4.23 ± 0.55	878 ± 50

Table II.2. Root:leaf ratios of *Salix nigra* cuttings grown at three nutrient levels and exposed to filtered air or low (0.06 ppm increased to 0.12 ppm) or high (0.12 ppm increased to 0.24 ppm) O_3. Values are means (n=6 for controls, n=12 for O_3-treated cuttings) ± standard errors.

O_3 Treatment	Low Nutrient	Medium Nutrient	High Nutrient
Control	1.30±0.25	1.54±0.21	0.95±0.11
Low O_3	1.59±0.16	1.39±0.14	1.07±0.13
High O_3	1.69±0.18	1.19±0.12	1.25±0.11

Table II.3. Regression statistics for plots of photosynthesis versus conductance for leaves of nodulated *Alnus serrulata* seedlings.

Treatment	r^2	alpha	Slope	Y-Intercept
Controls, Nodulated	0.617	0.025	18.18	3.53
Controls, Unnodulated	0.692	0.01	31.01	-2.02
O_3-treated in filtered air, Nodulated	0.654	0.025	40.35	-3.15
O_3-treated in filtered air, Unnodulated	0.323	0.25	13.26	-1.55
O_3-treated in O_3, Nodulated	0.167	0.50	-12.35	10.89
O_3-treated in O_3, Unnodulated	0.400	0.10	-6.55	1.88

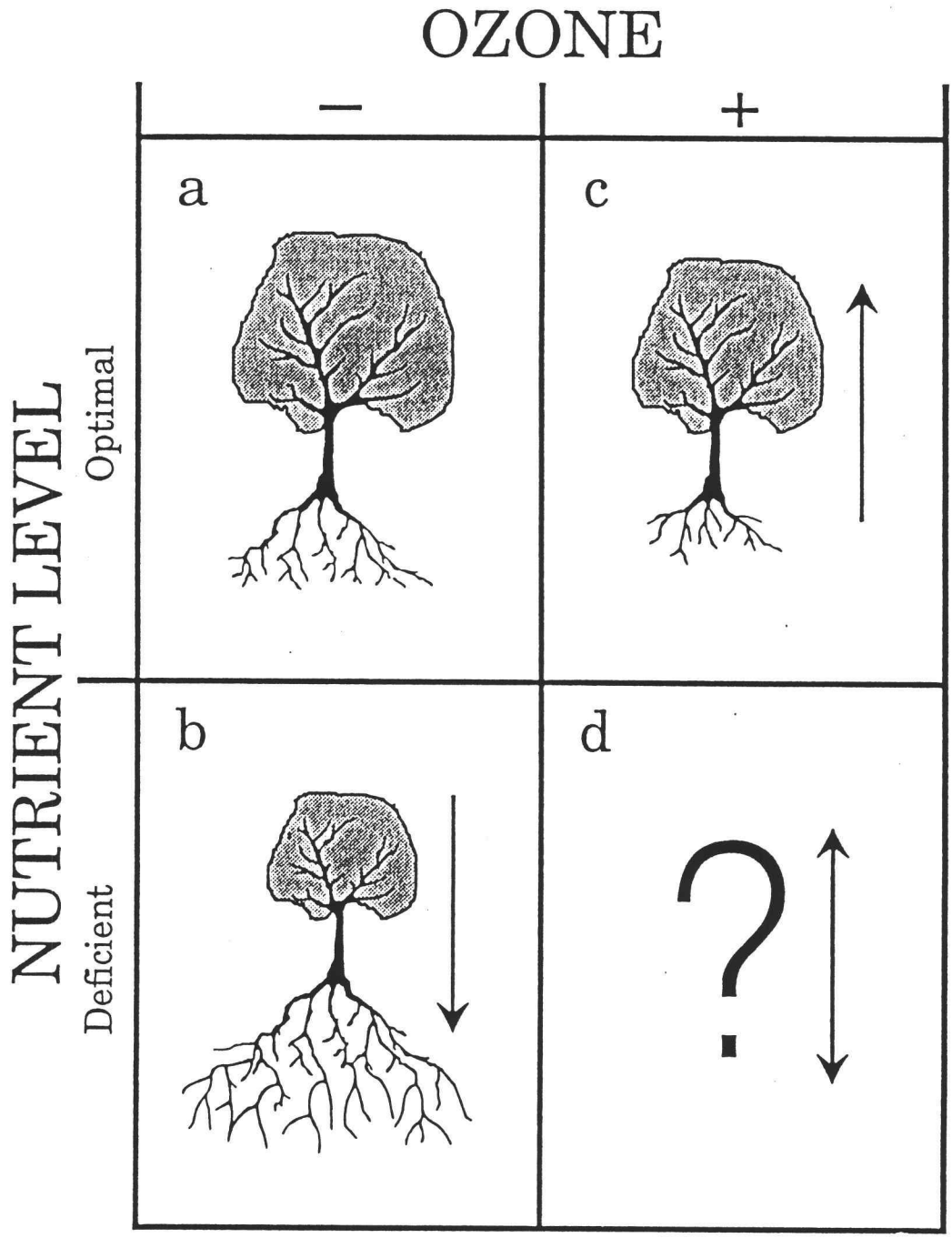

Figure II.1. Hypothesized changes in plant growth form with nutrient deficiency and/or ozone exposure. Arrows indicate direction of allocation shifts in response to stress. See text for details.

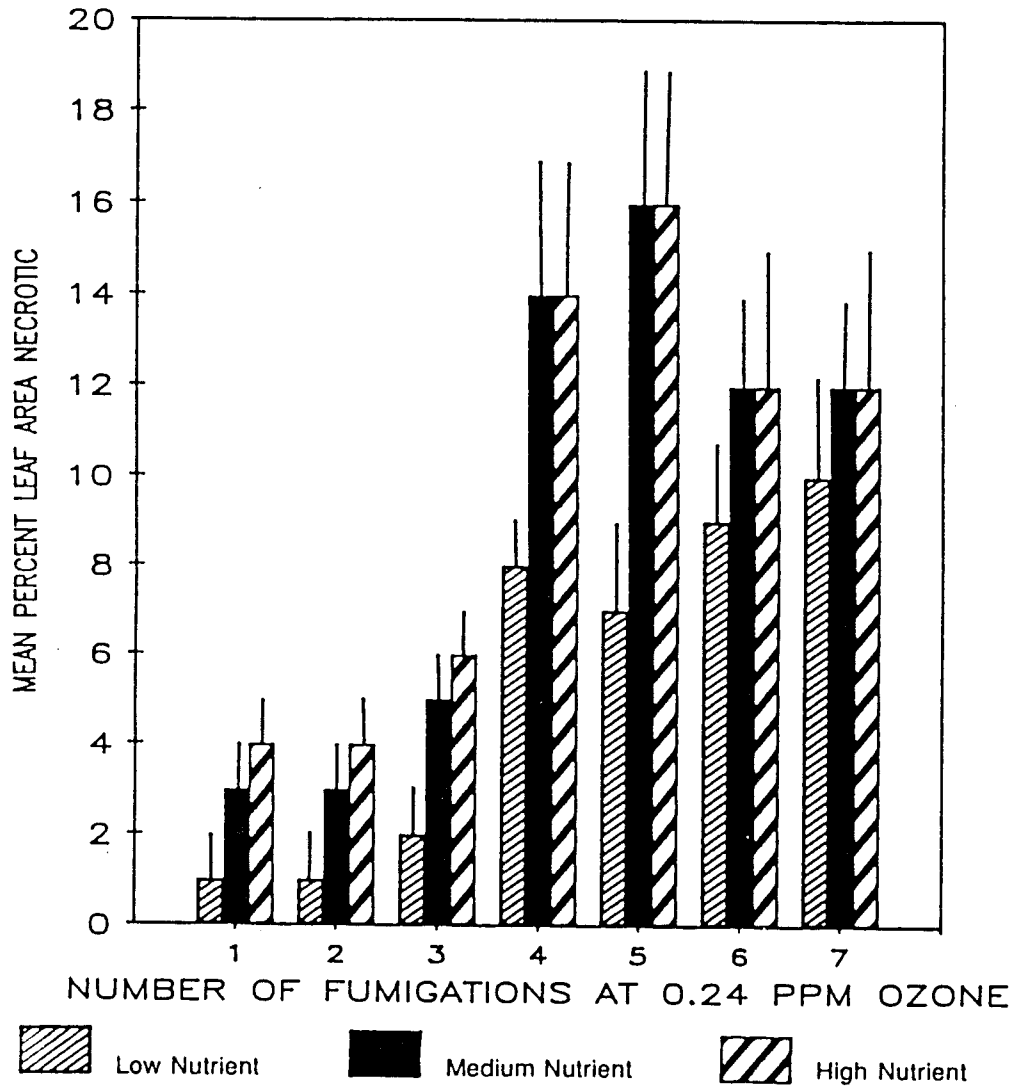

Figure II.2. Influence of nutrient status on percent leaf area necrotic in *Salix nigra* cuttings after 11 four h exposures to ozone at 0.12 ppm and a series of four h exposures at 0.24 ppm. Each bar represents the mean of 12 plants (± SE).

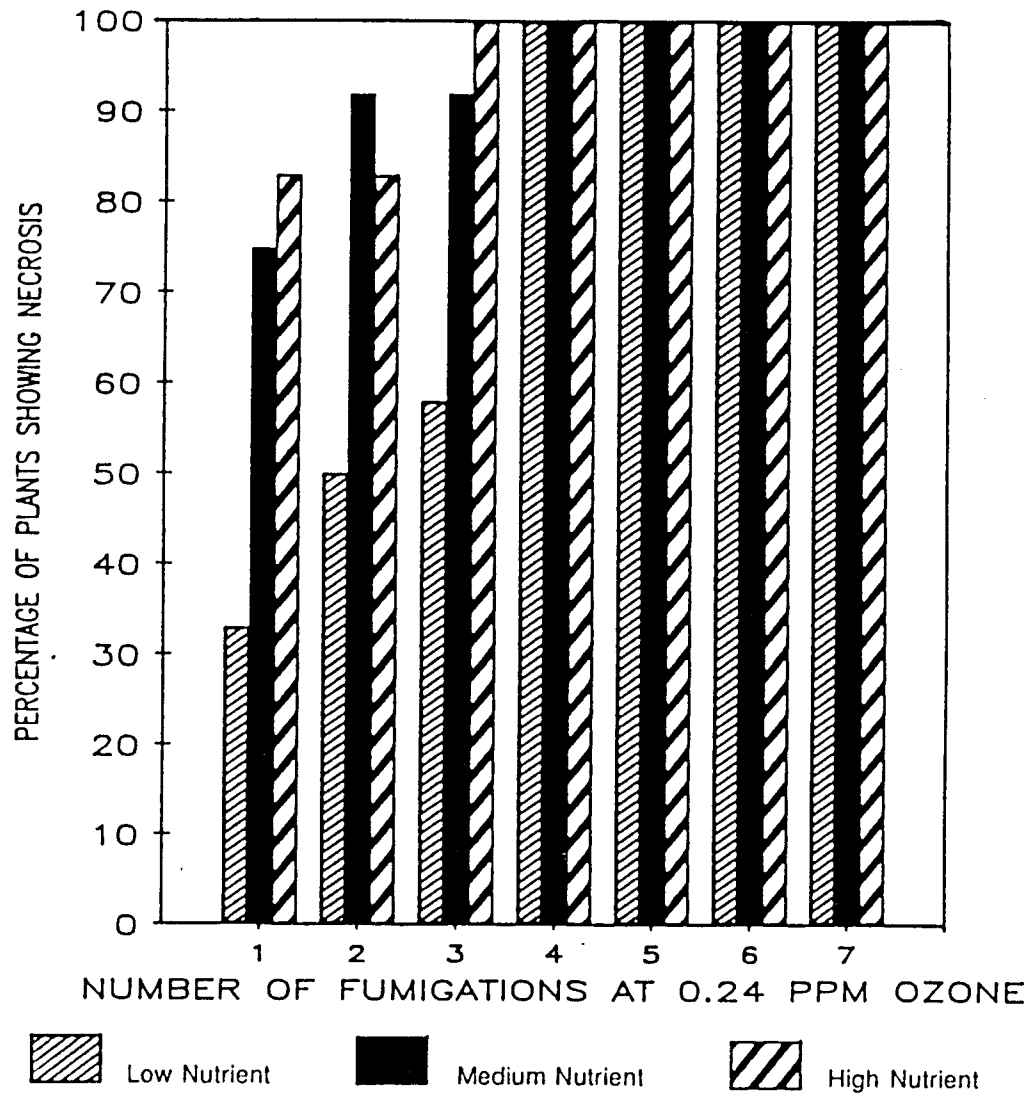

Figure II.3. Influence of nutrient status on the percentage of *Salix nigra* cuttings (N=12) showing necrosis after 11 four h exposures to ozone at 0.12 ppm and a series of four h exposures at 0.24 ppm.

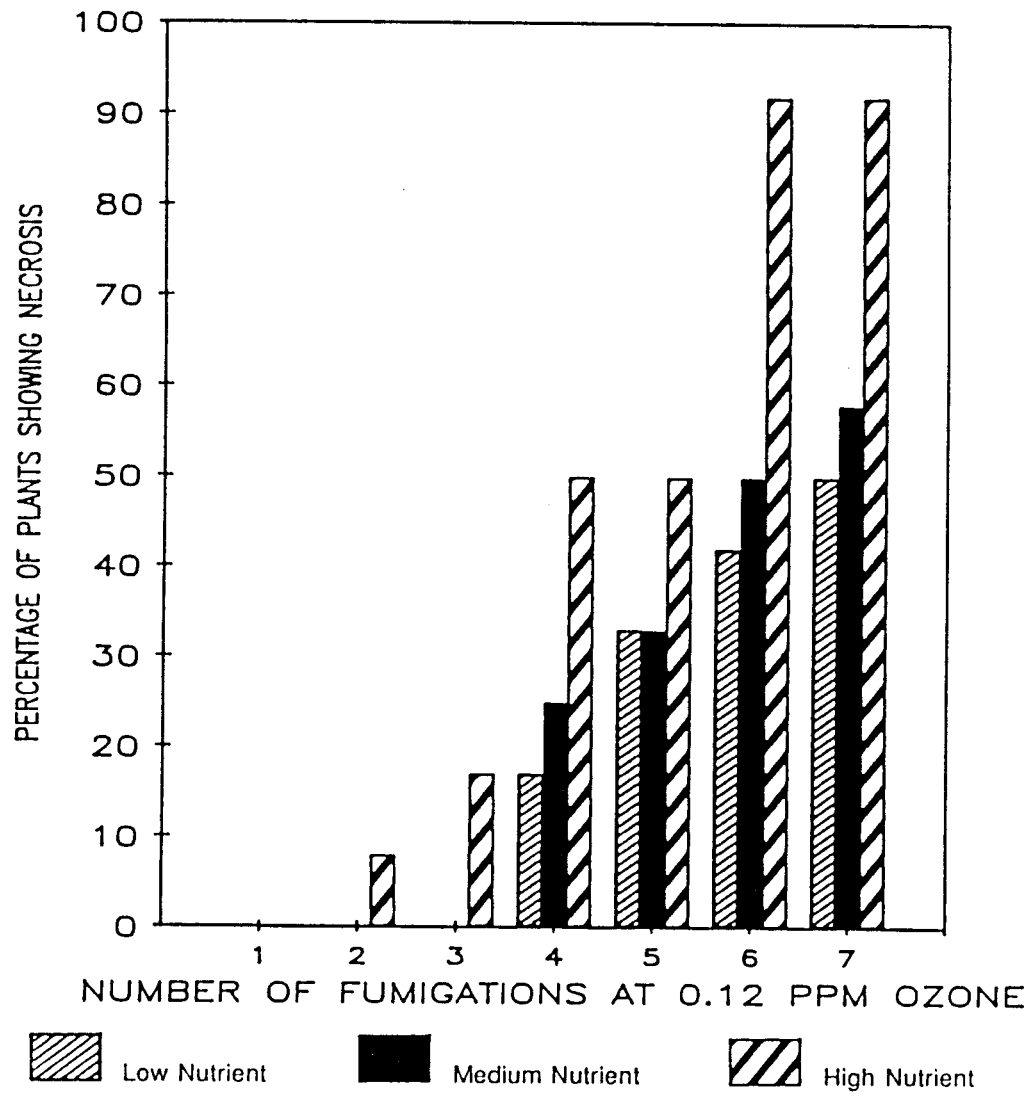

Figure II.4. Influence of nutrient status on the percentage of *Salix nigra* cuttings (N=12) showing necrosis after 11 four h exposures to ozone at 0.06 ppm and a series of four h exposures at 0.12 ppm.

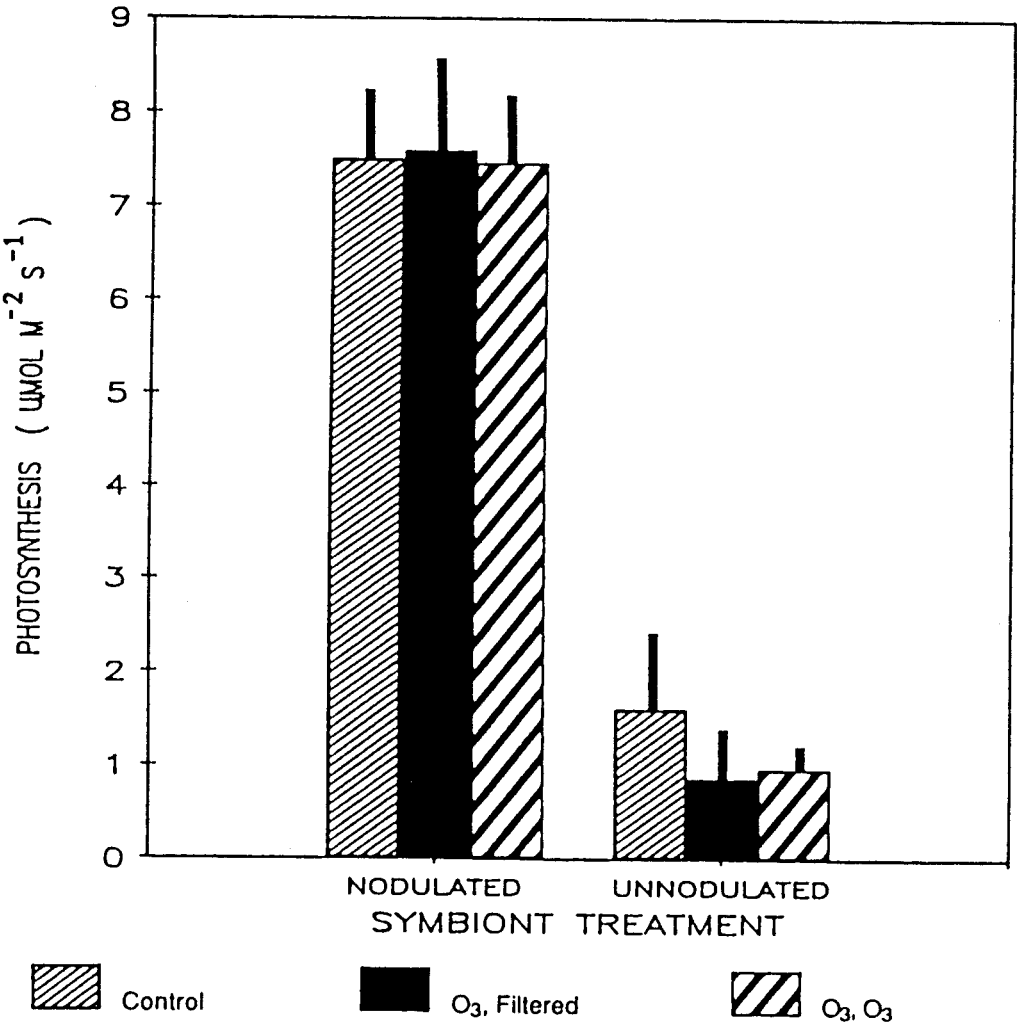

Figure II.5. Photosynthesis of nodulated and unnodulated *Alnus serrulata* seedlings exposed to filtered air (control) or 0.12 ppm ozone. Ozone-treated plants were measured in filtered air and in ozone. N=16 for controls; N=8 for ozone-treated. Bars are means ± SE.

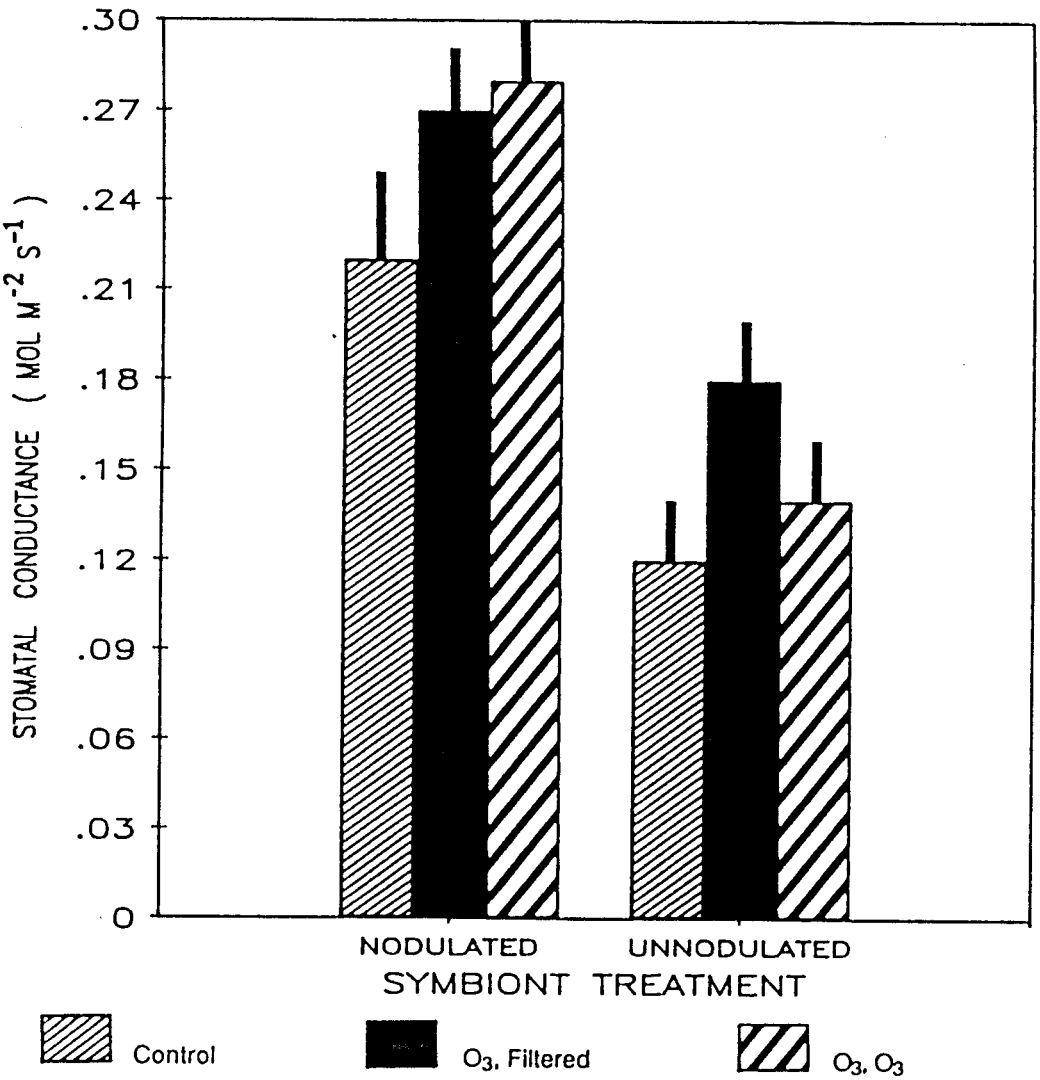

Figure II.6. Stomatal conductance of nodulated and unnodulated *Alnus serrulata* seedlings exposed to filtered air (control) or 0.12 ppm ozone. Ozone-treated plants were measured in filtered air and in ozone. N=16 for controls; N=8 for ozone-treated. Bars are means ± SE.

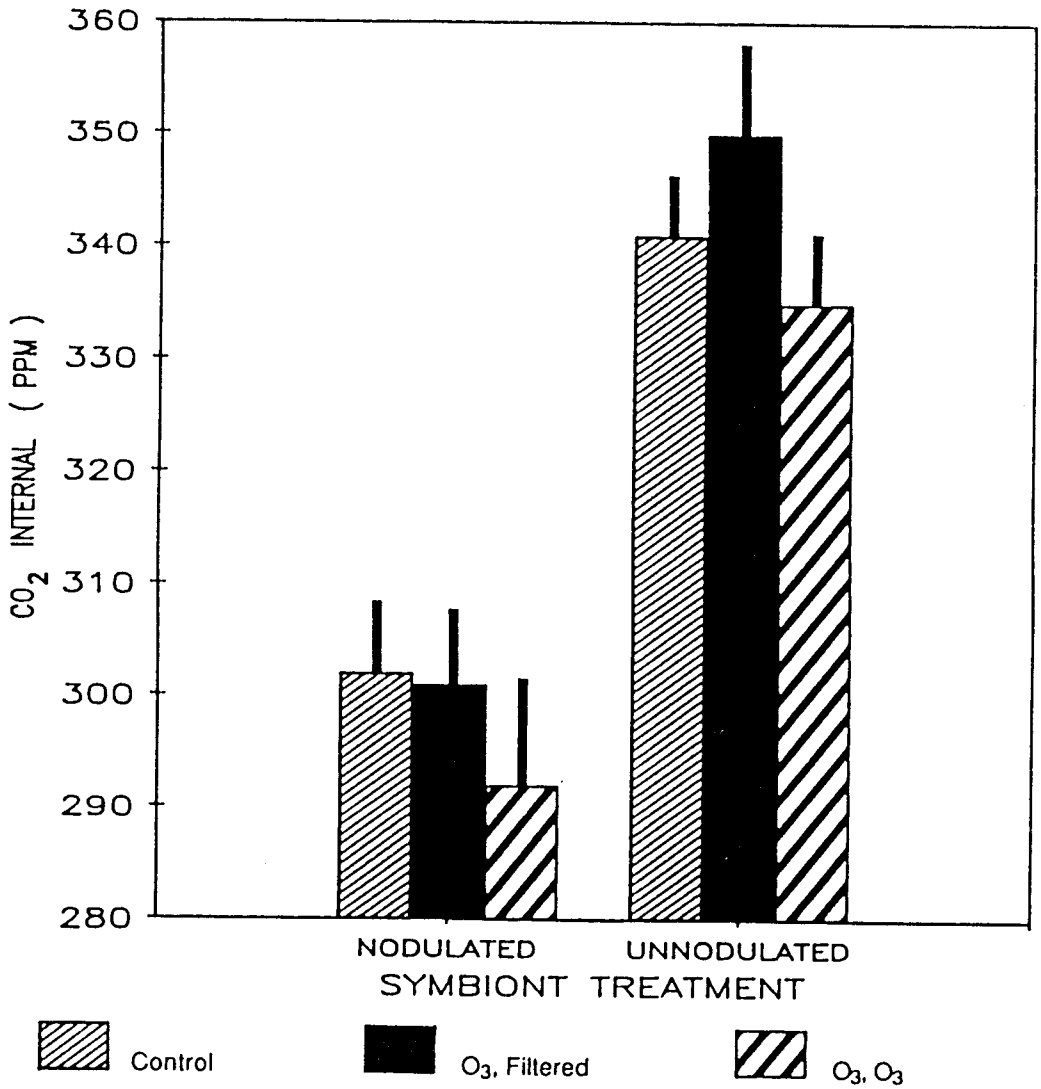

Figure II.7. Internal CO_2 concentration of nodulated and unnodulated *Alnus serrulata* seedlings exposed to filtered air (control) or 0.12 ppm ozone. Ozone-treated plants were measured in filtered air and in ozone. N=16 for controls; N=8 for ozone-treated. Bars are means ± SE.

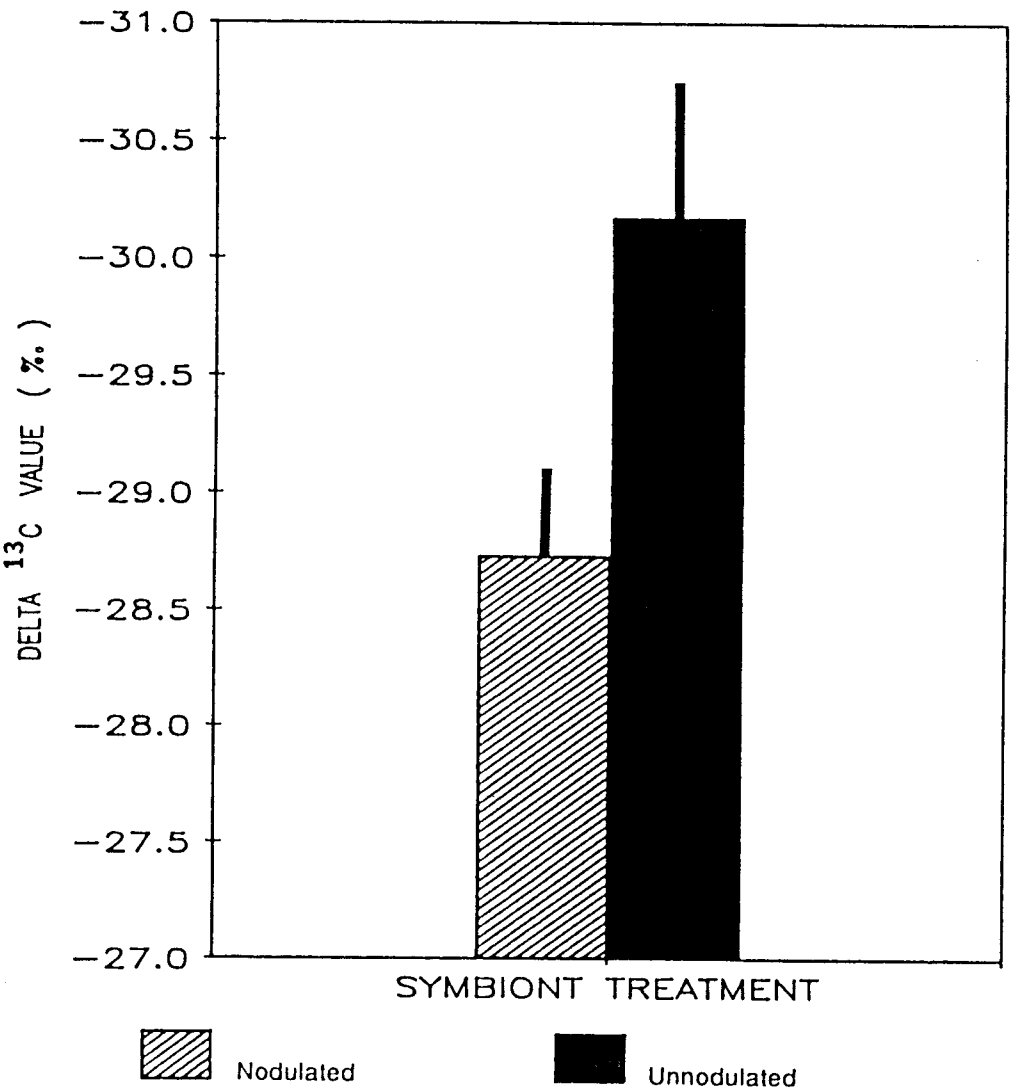

Figure II.8. Delta ^{13}C values of foliage of nodulated and unnodulated *Alnus serrulata* seedlings. N=4; bars are means ± SE.

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III. EFFECTS OF O_3 ON ALDER PHOTOSYNTHESIS AND SYMBIOSIS WITH *FRANKIA*

Greitner, C. S. & Winner, W. E. (1989a). Effects of O_3 on alder photosynthesis and symbiosis with *Frankia*. *New Phytologist* 111, 647-656.

SUMMARY

Alnus serrulata (Aiton) Willdenow seedlings with and without root nodules formed by the nitrogen-fixing actinomycete *Frankia* were exposed to clean filtered air or ozone (O_3) at 0.12 ul l^{-1} for 27 d (approximately 164 h total exposure). Gas exchange measurements on leaves and transmission electron micrographs of root nodule cells were made to detect any O_3 effects on the functioning of leaves and the root symbiont.

Photosynthesis, stomatal conductance, and internal CO_2 concentration were calculated for all plants in clean O_3-free air more than three weeks after the fumigations began. Significant positive correlations between photosynthesis and conductance were found for leaves of control nodulated and unnodulated alders and O_3-treated nodulated alders. There was a weak positive correlation between photosynthesis and conductance for unnodulated O_3-treated seedlings measured in clean air. When O_3-treated leaves were measured during fumigation with O_3, no positive correlation between photosynthesis and conductance was found for either nodulated or unnodulated seedlings. Photosynthetic rates of leaves having the highest stomatal conductance values were decreased by O_3 for both nodulated and unnodulated plants.

Transmission electron microscopy (TEM) revealed that after a 27 d exposure of shoots to O_3, host root cells of nodules from O_3-treated plants lacked organelles and showed extensive cytoplasmic breakdown. Hyphae and N_2-fixing vesicles of *Frankia* appeared normal. The *Frankia* endophyte seems to be more resistant to O_3 than is the host root nodule cell. These results show that ambient levels of O_3 may reduce photosynthesis and bring about associated degradation in rhizosphere symbiosis.

Key Words: O_3, *Frankia*, alder, rhizosphere symbionts, TEM.

INTRODUCTION

O_3 is a regionally distributed air pollutant that adversely affects plant growth (e.g. Reich and Amundson, 1985). Growth suppression results from foliar absorption of O_3 which subsequently changes metabolic processes in shoot tissue. Declines in photosynthesis may result directly from O_3 absorption and subsequent damage to components of mesophyll cells that play a role in photosynthesis. For example, O_3 may inhibit activity of ribulose-1,5-bisphosphate carboxylase (RuBisco) (Pell & Pearson, 1983) and result in increases in damaging free radicals (references in Heath, 1980; Tingey & Taylor, 1982). O_3 may also indirectly reduce photosynthesis by causing stomatal closure. Such effects of O_3 on photosynthesis have been reported for many species including coniferous and deciduous trees and crops (e.g. Reich & Amundson, 1985) at concentrations between 0.06 and 0.12 ul l^{-1}, values common throughout much of Europe and North America (Becker *et al.*, 1985).

Changes in metabolism of shoots are known to affect roots; altered carbon allocation patterns caused by O_3 result in decreased root:shoot ratios (Tingey, Heck & Reinert, 1971; Walmsley, Ashmore & Bell, 1980; Reinert, Shriner & Rawlings, 1982). This shift in root:shoot ratios has been interpreted as the consequence of reduced carbohydrate supplies for the plant and decreased assimilate translocation to the root (Tingey, Heck & Reinert, 1971). Studies with rhizosphere symbionts support the idea that O_3 suppresses translocation because O_3 fumigations decrease mycorrhizal infection and growth (McCool, Menge & Taylor, 1979; McCool & Menge, 1984) and reduce nodulation in the soybean-*Rhizobium* system (Manning *et al.*, 1971; Tingey & Blum, 1973; Blum & Tingey, 1977). Although the effects of O_3 on rhizosphere symbionts are likely to be mediated by changes in whole plant carbon metabolism, few studies provide links between changes in shoot physiology caused by O_3 and symbiotic relationships in the rhizosphere.

The effect of O_3 on rhizosphere symbiosis is important not only as an indicator of changes in whole plant carbon metabolism but

also because most plants rely on symbionts to augment nutrient availability. Mycorrhizae aid in uptake of nutrients, particularly phosphorous (Mejstrik & Benecke, 1969; Hayman & Mosse, 1972; Smith *et al.*, 1986), and nitrogen-fixing symbionts such as *Rhizobium* and *Frankia* can contribute significant quantities of nitrogen to their hosts (references in Bond & Wheeler, 1980; Jensen, 1986). Air pollutants that reduce the numbers and/or impair the functioning of rhizosphere symbionts would be particularly detrimental under nutrient-limited conditions. Thus air pollutants might directly reduce carbohydrate supply and indirectly induce deficiencies of P and N by injuring the rhizosphere symbioses.

In this study alder (*Alnus serrulata* (Aiton) Willdenow) growing with and without *Frankia*, an N_2-fixing root endophyte, was used to probe the effects of O_3 on both shoot physiology and rhizosphere symbiosis. The objective of this study was to examine the impact of O_3 on the alder-*Frankia* symbiotic relationship using techniques for measuring photosynthetic rates and analyzing root ultrastructure.

MATERIALS AND METHODS

Plant culture and inoculation

Alnus serrulata cones were collected from a single clump of stems in the Jefferson National Forest, Montgomery County, Virginia, in November 1985. Seeds were removed from cones, surface sterilized in household bleach (5.25% sodium hypochlorite) for 5 - 10 min, rinsed in deionized H_2O, and soaked in deionized H_2O for 3 d. Seeds were drained, placed in a plastic bag, and stratified for 41 d in a cold room (about 4 °C) followed by 3 d in a freezer (about -10 °C).

Seeds were sown in pots containing a 3:1 v:v mixture of vermiculite and clay in an O_3-free greenhouse. A 1,000 W metal-halide lamp provided supplemental lighting in the late afternoon to make a 16 h day/8 h night photoperiod. Seedlings emerged after several weeks and were watered daily. About 3 mo after sowing,

seedlings were transplanted to individual 0.7 l pots containing fresh potting mix and 0.5 g Osmocote 14-14-14 slow release fertilizer.

Five and a half months after planting, 17 seedlings were selected to be inoculated with *Frankia*. Root sections containing nodules were excised from alders in the field and brought to the lab where nodules were cut away from uninfected root tissue. Nodules were washed in deionized H_2O, blotted dry, weighed, surface sterilized in bleach for 15 min, and rinsed in deionized H_2O. The nodules were ground with a mortar and pestle, with the addition of sufficient deionized H_2O to make a 0.07 g ml^{-1} suspension. Two ml of the suspension were injected into the potting mix in several locations near the roots in each of the pots. Seventeen other seedlings received deionized H_2O instead of the nodule suspension. By approximately 8 months after sowing (2.5 mo after inoculation), new leaves of the successfully nodulated plants were observed to be larger and greener than the foliage of comparable unnodulated plants.

O_3 exposures and gas exchange measurements

Fumigations began in continuously stirred tank reactor (CSTR) fumigation chambers in the laboratory 8.5 mo after seeds were sown. Exposures to either 0.12 ul l^{-1} O_3 or O_3-free air were administered for 27 out of 29 days for periods ranging from 4 to 11 h per day. Fumigated plants were exposed to O_3 for a total of approximately 164 h. Light in the CSTR chambers was supplied by 1,000 W metal-halide lamps at about 400 - 500 uE m^{-2} s^{-1} . Plants were returned to the lighted greenhouse bench following each fumigation to maintain the 16 h daylight regime. Eight chambers were used for the study (four O_3 and four O_3-free control) and one pot each of nodulated and unnodulated alder was selected for treatment in each chamber.

A dynamic system for gas exchange measurement (LI-COR photosynthesis system, model LI-6200, 1 l cuvette) was operated inside the fumigation chambers, which had doors equipped with gloves for cuvette manipulation. Gas exchange measurements were made 24

and 29 d into the fumigation period, with the two days' data combined and analyzed together. A young, fully expanded leaf of each control and O_3-treated seedling was measured when plants were in the chambers in filtered air between fumigations (a total of four readings per treatment taken on each of the two measurement days). Leaves of O_3-treated plants were measured again at least one hour after exposure to O_3 began. Photosynthesis (*A*), stomatal conductance (g), and internal CO_2 concentration (C_i) were calculated by the system software. Ambient CO_2 concentrations were less than 387 ul l^{-1} before measurements were taken. Means and standard errors for the eight readings for each treatment were calculated, with significant differences based on the standard error of the mean. Linear regressions were calculated for plots of *A* versus g for each treatment.

Ultrastructure studies

At the conclusion of the O_3 exposure phase of the study, nodulated alder roots were washed free of potting medium. Nodules from the four O_3-treated plants were excised and combined, as were the controls. Nodules were cut into pieces in cold pH 6.9 paraformaldehyde glutaraldehyde acrolein (PGA) 3% fixative in 0.1 M cacodylate buffer. Samples were placed in vials of fresh fixative solution and stored under refrigeration (5°C) for several months.

Following an ethanol dehydration series, nodule pieces were embedded in Spurr's epoxy resin and sectioned on a Sorvall Porter Blum ultramicrotome. One um thick sections of the whole nodule area about 1 mm distal from the nodule tip were stained with toluidine blue and observed under a light microscope. Areas of equal development from near the youngest infected parts of the nodules were selected for both control and O_3-treated tissue. Ultrathin sections were stained with lead citrate and uranylacetate and observed with a Zeiss 10-CA TEM operated at 60 KV.

RESULTS

Effects of O_3 on shoots

Mean values of gas exchange parameters measured on leaves of nodulated and unnodulated alders were compared within O_3 treatments. In every case, unnodulated alders had significantly lower *A* and g values than did their nodulated counterparts (Table III.1). Thus unnodulated plants also had higher C_i values; mean C_i ranged from 292 to 301 ul l^{-1} for nodulated plants and 335 to 350 ul l^{-1} for unnodulated plants. Plants lacking the N_2-fixing nodules were visibly chlorotic and stunted compared with nodulated plants. The fact that nodulated plants were greener and had nearly four times the photosynthetic capacity of unnodulated plants is not surprising since *A* is known to increase with foliar N content for many species (Field & Mooney, 1986).

The O_3 exposures did not appear to affect the mean photosynthetic rates for nodulated plants (Table III.1). This was unexpected, since O_3 exposures of 0.15 ul l^{-1} or less are known to reduce photosynthesis in several tree and crop species (Reich & Amundson, 1985, and references therein). In addition, conductance of nodulated plants was increased about 20% by the fumigations. This increase in conductance was observed both during and between treatments. Normally, factors that increase conductance make CO_2 more available to mesophyll cells and photosynthesis also increases. Since this did not happen, one O_3 effect was to reduce water use efficiency.

Mean photosynthetic rates of unnodulated plants were reduced by O_3 by a small amount in absolute terms but a large amount in relative terms (Table III.1). Although the O_3 effects were not statistically significant, mean photosynthetic rates of leaves treated with O_3 were about 40% lower than for control leaves. As with nodulated plants, patterns of changes in photosynthesis caused by O_3 did not match patterns of changes in conductance.

Wong, Cowan & Farquhar (1979) observed that photosynthetic capacity and conductance were closely and positively correlated in

Zea mays when photosynthetic capacity was varied by altering nitrogen nutrition. Lange, Beyschlag & Tenhunen (1987) cited several studies showing a linear relationship between CO_2 assimilation and conductance. To test whether such a linear relationship existed for alder in the present study, *A* was plotted against g for leaves of control plants and plants that had received O_3.

When gas exchange rates of nodulated seedlings were measured in filtered air, both control (Fig. III.1a) and O_3-exposed (Fig. III.1b) leaves had significant positive correlations (at alpha=0.025 or less) between *A* and g. Regression analysis showed that these data sets fit straight lines. Similar plots of *A* and g were made for leaves of O_3-fumigated plants during exposures. No significant correlation was found for nodulated plants (Fig. III.1c). The scatter in this data set resulted primarily from leaves which had high g but did not have high *A* values. Thus the effects of O_3 on this regression were apparent only during fumigations and the relationship between *A* and g for O_3-treated leaves recovered in O_3-free air.

Control leaves of unnodulated plants also showed a significant positive correlation between *A* and g (alpha=0.01; Fig. III.2a). *A* and g were only weakly correlated (alpha=0.25) for O_3-treated leaves of unnodulated plants measured in filtered air (Fig. III.2b). In this case, poor nutritional status may have reduced the capacity for leaves to return to the expected relationship between *A* and g. When O_3-exposed leaves of unnodulated alder were measured in the presence of O_3, there was no positive correlation between *A* and g (Fig. III.2c). Lack of positive correlation was again due to leaves that had high g but did not have high *A* values.

Sensitivity to O_3 was associated with high conductance for leaves of both nodulated and unnodulated plants, presumably because leaves with high g absorb more air pollutant. In addition, leaves with high g or *A* are more metabolically active than are leaves with low g or *A* and high rates of metabolism may confer sensitivity to air pollutants (Winner & Mooney, 1980a). If the observation that

the leaves with highest metabolic rates are also most sensitive physiologically to O_3 is generally true, then it would appear that those leaves contributing most towards productivity are most vulnerable to O_3.

Effects of O_3 on root nodules

Since leaves with highest conductance had lower rates of photosynthesis than expected, the O_3 treatments may have altered not only rates of carbon gain but also patterns of whole plant carbon allocation. If declines in photosynthesis resulted in decreased carbon allocation to roots, the symbiotic relationship between the host root cells and *Frankia* could be affected. To test this idea, analysis of the ultrastructure of root nodule cells was conducted. Micrographs of *A. crispa* and *A. glutinosa* root nodules published by Lalonde & Knowles (1975a, b) and Lalonde (1980) were studied for interpretation of the structures observed in these *A. serrulata* micrographs.

Nodule cells from both control and O_3 treatments harbored numerous *Frankia* hyphae and septate vesicles (Fig. III.3a and b). Control nodule cells had evenly-grained cytoplasm and contained mitochondria, amyloplasts with starch grains, and vacuoles (Fig. III.3a). In contrast, nodule cells from an O_3-treated plant showed degradation of the cytoplasm and lacked mitochondria, amyloplasts, and vacuoles (Fig. III.3b). Mesosomes and nuclear material were visible in hyphae and in vesicles from each treatment (Fig. III.4a and b). The capsules that surrounded the endophyte were clearly bordered by host plasmalemma in control nodule cells (Fig. III.4a), but the host plasmalemma appeared to be disrupted in nodule cells from an O_3-exposed alder (Fig. III.4b).

Although some *Frankia* hyphae appeared singly, most occurred as many individuals packaged together in a capsule. This was true for both O_3-treated and control seedlings (Figs. III.3a and b) and is in contrast to that of *Frankia* symbioses with other hosts, where each hypha regularly has its own capsule (Iain Miller, personal communication). A hypha in a control nodule was observed between

two cells, and the capsule appeared to be continuous with the cell walls (Fig. III.5a). Hyphae were observed between the host cell walls of two nodule cells from an O_3-exposed plant (Fig. III.5b). O_3 did not affect *Frankia* in appearance or quantity of vesicles, hyphae, mesosomes, and nuclear material, or its packaging in capsules or movement of hyphae from cell to cell.

DISCUSSION

Effects of O_3 on the relationship between photosynthesis and conductance

Leaves of control nodulated and unnodulated alder and O_3-treated nodulated alder measured in O_3-free air showed significant positive correlations between conductance and photosynthesis. Exposure to O_3 altered this relationship. The departure from linearity observed in nodulated alder during fumigation with O_3 was caused mainly by the leaves with the highest conductance values.

Absorption rates of gaseous pollutants are known to increase with g (Winner & Mooney, 1980a, b; Reich & Amundson, 1985), which is consistent with the idea that leaves with high conductance are more vulnerable to gaseous pollutants. Thus factors which reduce conductance may serve to coincidentally protect plants from air pollution absorption. Environmental stresses known to reduce g include drought, nutrient deficiency, shade (Jones, 1983), heat (under certain conditions: Berry & Bjorkman, 1980), and elevated CO_2 (Strain, 1987). Physiological changes associated with aging (Solarova, 1980) also reduce g. Photosynthesis is positively correlated with both foliar nitrogen and g (Field & Mooney, 1986). Thus rhizosphere symbionts may act initially to increase foliar nutrient contents and thereby increase both *A* and g. In support of this hypothesis, the nodulated alders in this study had significantly higher *A* and g and lower C_i values than did unnodulated plants. It follows then that symbionts may increase air pollution absorption capacity for plants.

The relationship between photosynthetic capacity and air pollution sensitivity is not clear. Some studies show that leaves or species with high photosynthetic capacity are more sensitive to pollutants such as SO_2, as measured by photosynthetic response, than leaves with low photosynthetic capacity (Winner & Mooney, 1980a). On the other hand, growth of ryegrass is known to be more sensitive to SO_2 during winter periods when growth is slow (Bell & Clough, 1973). This study with alder contributes information about the relationship between metabolic rates and air pollution sensitivity of leaves but was not designed to resolve whether the effects of O_3 on leaves with high g were due to high O_3 absorption or high sensitivity to absorbed O_3.

Finally, analysis of these gas exchange data shows that the method of simply comparing mean *A* and g values for fumigated and control leaves may be an unsatisfactory approach for physiological studies. The effect of O_3 in this study was not apparent until *A* and g were plotted and analyzed by correlation and regression. Important trends revealed by these analyses were not evident in the calculation of mean values for treatments.

Effects of O_3 on ultrastructure of the rhizosphere symbiosis

As O_3 does not penetrate plant potting media to any extent (Blum & Tingey, 1977), it is unlikely that O_3 directly affected the alder root nodules. Sections from bulk samples of whole nodules showed no ultrastructural differences between nodules from control and O_3-exposed plants (micrographs not shown). Sections taken from near the tips, the younger parts of the nodules, did show great differences between the two treatments (Figs. III.3-5). Presumably these areas were developing and growing under O_3 stress whereas the older areas (where vesicle breakdown, a natural part of nodule aging, was common for both control and O_3-treated plants) had completed development before O_3 stress began. O_3 reduced photosynthesis in leaves having the highest conductance values, indicating that carbon gain for the plant as a whole declined during fumigation. The death of host root nodule cells was most likely a

consequence of decreased availability of carbohydrates to roots induced by O_3.

The structural integrity of the endophyte was maintained in an O_3-treated plant whereas that of the host root nodule cells was not. There are several possible explanations. Perhaps *Frankia* hyphae and vesicles constitute a stronger sink for carbohydrates from the leaves than do the host root cells. Another possibility is that *Frankia*, but not host root cells, may become dormant when the nodule lacks carbohydrates as a result of O_3 stress to the shoots. Alternatively, reductions in assimilate availability to *Frankia* may result in the endophyte utilizing components of root cells as a carbon supply. Electron microscopy studies suggest that *Frankia* can produce enzymes that degrade cell wall materials (Lalonde & DeVoe, 1975; LaLonde & Knowles, 1975a, b; Berry, McIntyre & McCully, 1986). Thus the endophyte has the potential to acquire carbon from the host by digesting root tissue. If so, one effect of O_3 may be to change the alder-*Frankia* relationship form a symbiotic to a parasitic form.

Although the *Frankia* cells were not visibly affected by O_3 treatment, a fumigation regime of longer duration might result in structural damage to the endophyte. Degradation of the root cell cytoplasm would eventually interfere with the transfer of carbohydrates from the host to *Frankia*. The weakened nutritional status of the endophyte would also cause a decline in the rate of N_2 fixation. In addition the NH_3 which was produced by *Frankia* might not be absorbed or assimilated as root cells deteriorated. In this scenario, the N nutrition of the shoot would be adversely affected, resulting in decreased photosynthesis. Thus O_3 may reduce photosynthesis by direct damage to leaf cells and by indirect effects associated with disruption of the normal process of symbiotic N_2 fixation. Inhibition of root growth by O_3 for plant species lacking N_2-fixing organisms may also result in impaired N status. Reduction in carbon allocation to roots caused by O_3 is likely to result in decreased N absorption from the soil.

This study demonstrates an association between changes in shoot physiology induced by O_3 with changes in the structure of the

alder-*Frankia* symbiosis. The authors have not seen any other reports in the literature showing ultrastructural changes in root cells resulting from exposure of shoots to O_3. The extent to which the functional capacity of *Frankia* to supply N to alder was compromised as the result of O_3 treatments, and the effects of O_3 on assimilate translocation will be explored in future studies.

ACKNOWLEDGMENTS

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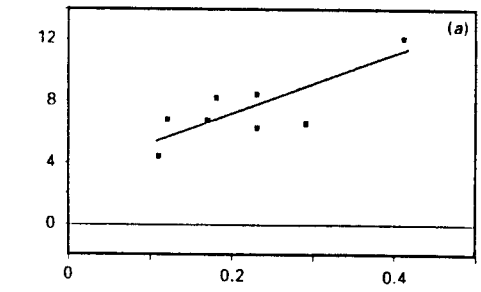
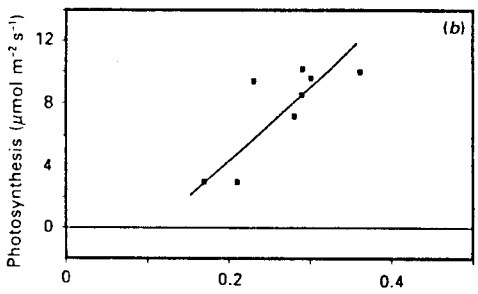
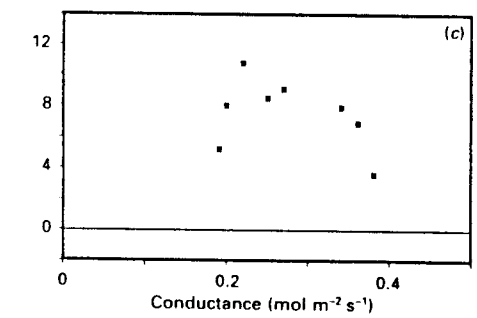
Table III.1. Gas exchange measurements* of *Alnus serrulata* leaves.

	Photosynth. (umol m^{-2} s^{-1})	Stomatal Conductance (mol m^{-2} s^{-1})
Controls, Nodulated	7.50 ± 0.74	0.22 ± 0.03
Controls, Unnodulated	1.60 ± 0.80	0.12 ± 0.02
O_3-treated in O_3-free air, Nodulated	7.59 ± 0.99	0.27 ± 0.02
O_3-treated in O_3-free air, Unnodulated	0.87 ± 0.49	0.18 ± 0.02
O_3-treated in O_3, Nodulated	7.46 ± 0.74	0.28 ± 0.02
O_3-treated in O_3, Unnodulated	0.97 ± 0.20	0.14 ± 0.02

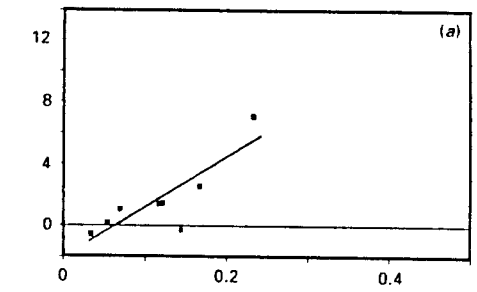
* Values are means ± standard errors (n = 8)

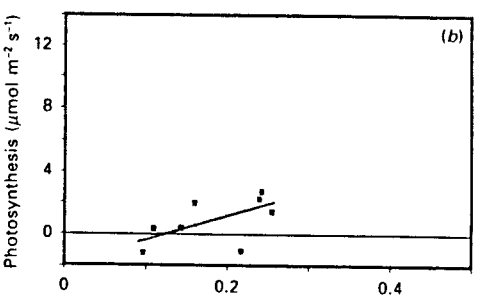


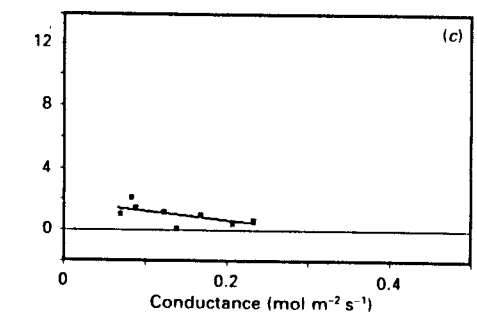




(Left) Figure III.1. (a-c) Plots of photosynthesis versus conductance for leaves of nodulated *Alnus serrulata* seedlings. (a) Control, with regression line drawn (r^2=0.617, significant at alpha=0.025; slope=18.18;Y intercept=3.53). (b) O_3-treated leaves in O_3-free air beween fumigations, with regression line drawn (r^2=0.654, significant at alpha=0.025; slope=40.35;Y intercept=-3.15). (c) O_3-treated leaves during fumigation with 0.12 ul l^{-1} O_3 (no significant correlation).







(Right) Figure III.2. (a-c) Plots of photosynthesis versus conductance for leaves of unnodulated *Alnus serrulata* seedlings. (a) Control, with regression line drawn (r^2=0.692, significant at alpha=0.01; slope=31.01;Y intercept=-2.02). (b) O_3-treated leaves in O_3-free air beween fumigations, with regression line drawn (r^2=0.323, significant at alpha=0.25; slope=13.26;Y intercept=-1.55). (c) O_3-treated leaves during fumigation with 0.12 ul l^{-1} O_3 with regression line drawn (r^2=0.400, significant at alpha=0.10; slope=-6.55;Y intercept=1.88).

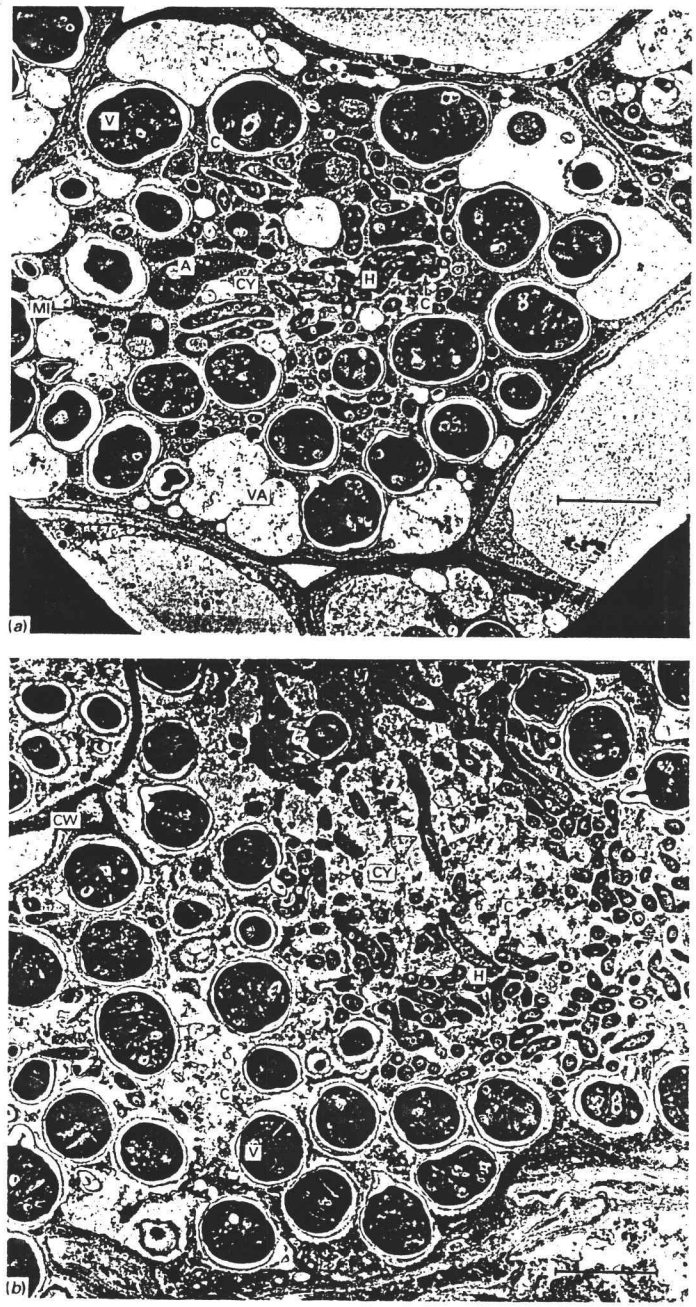


Figure III.3 (a,b) Root nodule cells from control (a), and O_3-treated (b), *Alnus serrulata* seedlings, showing endophyte hyphae (H) and vesicles (V), both surrounded by capsules (C). Note presence of amyloplasts (A), mitochondria (MI) and vacuoles (VA), and appearance of cytoplasm (CY) of control nodule cell, and absence of organelles and disruption of host cytoplasm in cell from an O_3-treated seedling. Bars are 5 um.

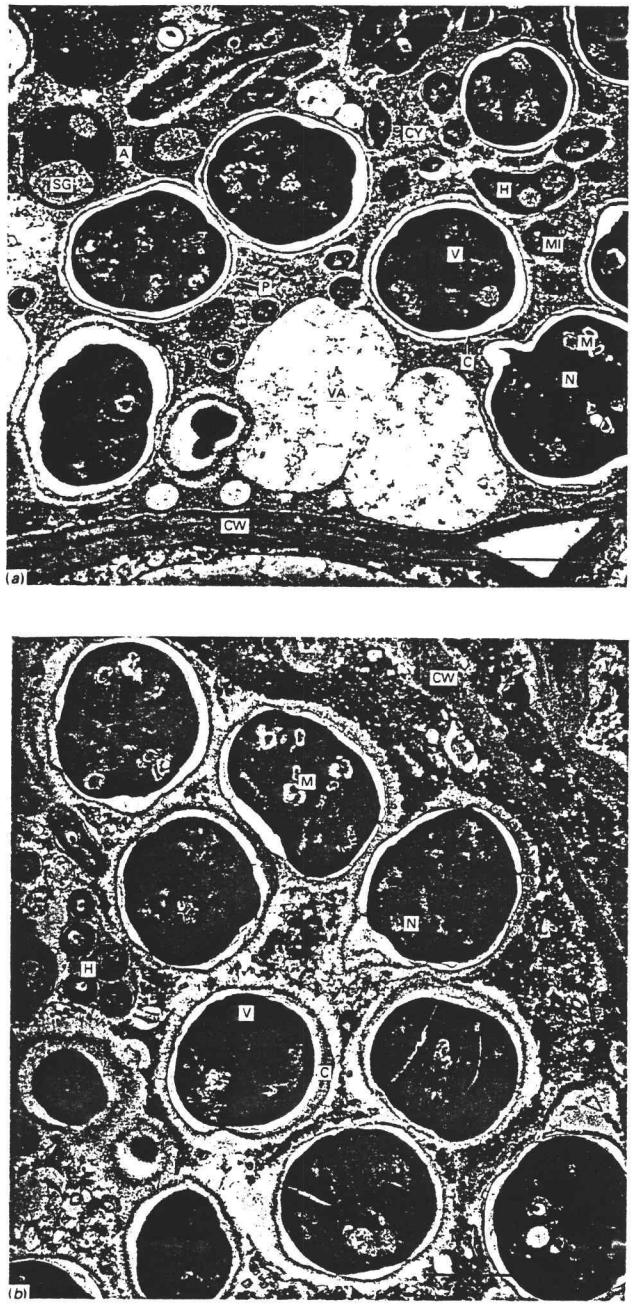


Figure III.4 (a, b) Details of Fig. III.3a, b) showing that hyphae (H) and septate vesicles (V) of the endophyte from both control (a), and O_3-treated (b), *Alnus serrulata* seedlings contain mesosomes (M) and nuclear material (N) and are surrounded by capsules (C). Amyloplasts (A) of the control cell contain starch grains (SG). Note degradation of host plasmalemma around capsules in the cell of the O_3-treated plant, contrasted with distinct membrane (P, arrow) seen in the control cell. Host cell wall, CW; other abbreviations as in Fig. III.3 (a, b). Bars are 5 um.

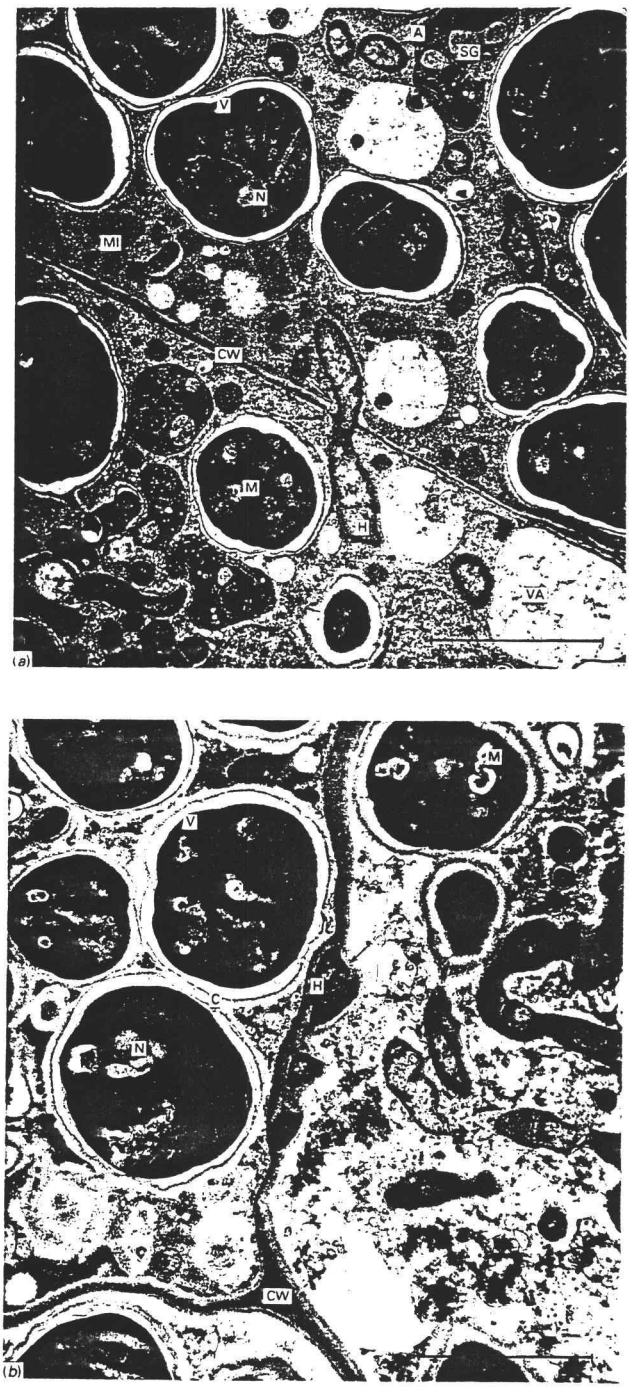


Figure III.5. (a,b) Root nodule cells from *Alnus serrulata* seedlings. Control root nodule cells (a) showing hypha (H) passing through the host cell wall (CW). Root nodule cells from O_3-treated plant (b), showing hyphae between host cell walls. Note continuity of cell wall and capsule (arrow) in both figures. Abbreviations as in Fig. III.3 (a, b), III-4(a, b). Bars are 5 um.

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IV. INCREASES IN DELTA ^{13}C VALUES OF RADISH AND SOYBEAN PLANTS CAUSED BY O_3

Greitner, C. S. & Winner, W. E. (1988). Increases in delta ^{13}C values of radish and soybean plants caused by ozone. *New Phytologist* **108**, 489-494.

SUMMARY

This greenhouse study was undertaken to determine whether air pollution-caused changes in photosynthesis and conductance also affected the delta ^{13}C value of plant tissue. Experiments with radishes (*Raphanus sativus* L. cv. Cherrybelle) and soybeans (*Glycine max* L. Merr. cv. Williams) exposed to ozone (O_3) at 0.12 ul l^{-1} showed significant growth reductions, 14 to 20 ul l^{-1} reductions in CO_2 internal (C_i), and +0.3 to +0.7‰ shifts in the delta ^{13}C values of leaves and roots. These results indicate that ambient O_3 may influence the delta ^{13}C values of plants found in many agricultural and industrial regions, and that O_3 may act to simultaneously suppress growth and increase water use efficiency (WUE) of plants. Stable carbon isotope techniques may be useful for integrating the metabolic effects of O_3 over long air pollution exposures.

Key words: Ozone, stable carbon isotopes, delta ^{13}C, radish, soybean.

INTRODUCTION

Analysis of the stable carbon isotopes ^{12}C and ^{13}C in plant tissue provides a historical record of important metabolic parameters summarized as delta ^{13}C values. Delta ^{13}C values are measurements of the extent to which the plant discriminated against the heavier ^{13}C during carbon fixation, with more negative values indicating greater discrimination. C_3 plants have delta ^{13}C values that typically range from -22 to -40‰ (Troughton, 1979). This range of values is attributable both to consistent differences among species and to the role of environmental factors on the physiological processes of plants. This study tested the idea that ambient concentrations of O_3, a gaseous air pollutant, cause shifts

in delta ^{13}C values of plants.

The capacity for ribulose bisphosphate carboxylase (Rubisco) in C_3 plants to preferentially fix $^{12}CO_2$, as opposed to $^{13}CO_2$, diminishes as the concentration of CO_2 in the leaf mesophyll (CO_2 internal or C_i) drops. A decline in C_i could occur when an environmental factor causes a large decrease in conductance and little or no change in photosynthesis. Thus, reduced C_i results in an overall increase in water use efficiency (WUE, photosynthesis transpiration^{-1} in mg CO_2 g^{-1} H_2O). Previous studies (Farquhar, O'Leary & Berry, 1982) have shown that environmental factors which bring a change in the C_i value will result in a change in isotope fractionation during carbon fixation. The effect of environmental factors on the relationship between C_i and delta ^{13}C values has been shown for plants exposed to water stress (Winter, 1981; Farquhar *et al.*, 1982; Farquhar & Richards, 1984).

O_3, a regionally distributed gaseous air pollutant, has generally been shown to decrease both photosynthesis and conductance (e.g. Reich & Amundson, 1985). Therefore, exposure to O_3 may also alter delta ^{13}C values of plants. To test this idea, plants were raised both with and without O_3, and gas exchange measurements and delta ^{13}C values were compared. Recognition of an isotopic shift would be important because it would show the long-term effects of O_3 on C_i that result from changes in photosynthesis and conductance. If O_3 resulted in changes in C_i, delta ^{13}C values might be useful for integrating O_3-caused changes in photosynthesis and conductance over time. There also exists the possibility that delta ^{13}C values could eventually be used as a bioindicator of O_3 stress.

The specific objectives of these experiments were to assess the effects of O_3 on plant growth, photosynthesis, conductance, C_i, and WUE, and to measure O_3-caused changes in delta ^{13}C values of plant tissues. These results are analyzed to show how delta ^{13}C values can be used to determine whether physiological responses of plants to O_3 measured over the short term were responses that were typical of the entire O_3 exposure period.

MATERIALS AND METHODS

Plant culture and exposure facilities

Radish (*Raphanus sativus* L. cv. Cherrybelle) and soybean (*Glycine max* L. Merr. cv. Williams) plants were raised from seeds in a potting medium of clay, vermiculite and a slow release inorganic fertilizer. Soybeans were thinned to four plants per pot, and radishes to five per pot. Plants were propagated in an O_3-free greenhouse until nine days after planting, then fumigations began in continuously stirred tank reactor (CSTR) laboratory fumigation chambers. Plants were watered daily and water stress was never apparent.

Exposures to either 0.12 ul l^{-1} O_3 or O_3-free air were administered for 20 out of 22 days for soybeans and 19 out of 20 days for radishes, with exposure periods ranging between four and 11 h per day. Soybeans were fumigated for a total of approximately 117 h and radishes for 106 h. This O_3 concentration was chosen because it can occur under ambient conditions. Eight chambers were used for the study (four O_3 and four control) and 15 pots of each species were treated in each chamber. Light in the CSTR chambers was about 800 uE m^{-2} s^{-1} and was supplied by 1,000 W metal halide lamps. Photoperiod in the greenhouse and chambers was 12 h days/12 h nights. Plants were fumigated in known intervals so that total exposure time as a fraction of growth period was known. Therefore, after fumigations began at age 9 d, plants were in the fumigation chambers exposed to either O_3 or filtered air for about 48% of the daylight period.

Gas exchange methods

Gas exchange data were recorded 28 days after planting for radishes and 30 days after planting for soybeans. Measurements were made with a portable device (LI-COR photosynthesis system, model 6200, with 1 l cuvette) operated inside the fumigation chamber. Chamber doors were equipped with gloves for cuvette and instrument manipulation. The LI-COR 6200 is a closed system that measures

changes in humidity and rates of CO_2 depletion. CO_2 drawdown rates by leaves resulted in measurement periods of 30 - 50 s.

Although some leaves of O_3 fumigated plants had stippling symptomatic of macroscopic injury, gas exchange measurements were taken on leaves free of these markings. Fully expanded radish leaves at age 25 d and age 19 d, and the first trifoliolate leaves of soybean, were measured for photosynthesis, conductance, and C_i. Four or five radish leaves of both ages and treatments and 15 soybean leaves of each treatment were measured. Ambient CO_2 concentrations were less than 380 ul l^{-1} before measurements were taken. Gas exchange parameters were computed using the LI-COR system datalogger and software. Mean photosynthesis, conductance, C_i values and standard errors were calculated. All measurements were made between 10:00 and 18:00, alternating readings in control and O_3 chambers. Leaves were measured when all plants were in filtered air before the fumigation began for the day (non-exposure period measurements), and again several hours after the fumigation started.

Harvesting and delta ^{13}C techniques

Soybean plants were harvested one day, and radishes two days after gas exchange data were taken. Plants were removed from pots and the potting medium was washed from the roots. Leaves, stems, and roots were separated, oven dried, and weighed to 0.001 g for growth analysis. Leaf areas were measured prior to drying with a LI-COR area meter. Samples were ground to pass a 40 mesh screen and sent for mass spectrographic analysis to Dr. James Ehleringer, University of Utah, Salt Lake City, Utah. Delta ^{13}C values of the tissues were determined by comparing the relative abundance of ^{13}C in tissue with the abundance of ^{13}C in the PeeDee Belemnite (PDB) standard using the following equation:

$$\text{delta } ^{13}C \text{ ‰} = \left[\frac{^{13}C/^{12}C \text{ in sample}}{^{13}C/^{12}C \text{ in standard}} - 1 \right] \text{X } 1000.$$

RESULTS

Growth responses

Ozone at 0.12 ul l^{-1} caused similar declines in leaf area and in plant dry weight for both radish and soybean plants (Table IV.1). Total leaf area was decreased by 12.0% for radishes and 9.5% for soybeans. Leaf dry weight declined by 9.6% and 11.6% for radishes and soybeans, respectively. Percent reductions in leaf area and dry weights were close in value, indicating that O_3 did not greatly affect leaf specific weight (weight $area^{-1}$).

Ozone caused greater declines in root (below ground tissues) dry weight than in shoot weight. Reduction of below ground dry weight was 26.2% for radishes and 24.3% for soybeans. Dry weight reductions of above ground tissues were 15.4% for radishes and 11.7% for soybeans. The root:shoot ratio was therefore decreased by 14.7% in radishes and 14.5% in soybeans. These patterns of growth response to O_3 are well known for both radishes (Tingey, Heck & Reinert, 1971; Walmsley, Ashmore & Bell, 1980; Reinert, Shriner & Rawlings, 1982) and soybeans (Reich & Amundson, 1985).

Physiological responses

Gas exchange measurements during non-exposure periods showed O_3-treated plants had photosynthesis, conductance, and C_i values which were not significantly different from those of controls. The results reported below were obtained at the time the O_3-treated plants were being exposed to O_3. Physiological responses to O_3 differed for the 19 and 25 d old radish leaves. Ozone decreased stomatal conductance by about 35% for both ages of radish leaves (Fig. IV.1). However, O_3 caused no change in photosynthesis of 19 day old leaves but a 37% reduction for 25 d old radish leaves (Fig. IV.2). Thus photosynthesis of younger radish leaves formed during exposure to O_3 stress was more resistant to this pollutant than was photosynthesis of older leaves formed initially in O_3-free air. Ozone also resulted in about a 35% decrease in conductance for soybean leaves with no apparent change in photosynthesis (Figs.

IV.1, 2).

C_i values were determined because they reflect both conductance and photosynthesis and indicate changes in plant WUE. C_i values diminished significantly in 19 day old (20 ul l^{-1} decrease) and 25 day old (15 ul l^{-1} decrease) radish leaves and in soybean leaves (14 ul l^{-1} decrease) exposed to O_3 (Fig. IV.3). Therefore, although O_3 reduced growth, reductions in C_i indicate that O_3-caused changes in conductance led to a general increase in WUE for soybean and 19 d radish leaves in this experiment.

Isotopic shifts

In order to determine whether changes in C_i, photosynthesis, and conductance measured near the harvest date were reflective of plant status throughout the experiment, stable carbon isotope ratios were measured. These data provided a way to integrate the effects of O_3 on C_i over the life of the plants.

Delta ^{13}C values were calculated for leaves and roots of soybeans and radishes (both age classes combined), and for radish hypocotyls (Fig. IV.4). In every case, O_3 caused an increase in delta ^{13}C values. The O_3 treatment resulted in leaves becoming more positive by 0.3‰ to 0.5‰. Expected shifts in delta ^{13}C values caused by C_i reductions of 14 and 20 ul l^{-1} were calculated using equations from Farquhar *et al.* (1982). A decrease of C_i by 14 ul l^{-1} should have resulted in a 0.9‰ increase, and by 20 ul l^{-1}, a 1.3‰ increase in the delta ^{13}C value, if plants were fumigated and had reduced C_i values during all of their growth. However, these plants may have had some periods when C_i values were not reduced by 14 - 20 ul l^{-1}. These periods could have been the nine days of growth prior to fumigations and/or days during which O_3 was only administered for 4 h. After fumigations began at age 9 d, plants were exposed to O_3 for about 48% of their total hours in daylight. Therefore, about 48% of the photosynthate produced during the fumigation phase of the study would be expected to have been altered in isotopic composition. About 52% of the photosynthate would have been unaffected, since C_i values for fumigated and control plants did not

differ during non-fumigation periods. Thus only 48% of the 0.9‰ and 1.3‰ delta ^{13}C shifts calculated from the Farquhar *et al.* (1982) equations should be expected. Such anticipated shifts would be 0.4‰ and 0.6‰ which approach the values actually measured. Even though there is still some discrepancy between expected and measured delta ^{13}C values, these stable isotope data indicate that O_3-caused shifts in C_i were persistent for much of the foliage throughout the O_3 exposures.

The O_3-caused increase in foliar delta ^{13}C values was also reflected in hypocotyl and root tissues of test plants. The below ground tissues of both control and O_3-treated plants had higher delta ^{13}C values than those of above ground tissues, perhaps related to isotopic fractionation by processes associated with translocating carbon from leaves to roots or by root metabolism. In either case, the magnitude of the difference between delta ^{13}C values of below and above ground tissues was not altered by O_3. These results suggest that the sensitivity of root growth to O_3 is actually manifest by O_3-caused changes in carbon metabolism in leaves and that O_3 has little direct effect on roots.

DISCUSSION

The increase in delta ^{13}C values upon treatment with O_3 apparently was correlated with the lowered C_i that resulted from the partial stomatal closure induced by this air pollutant. Presumably Rubisco is less discriminating against $^{13}CO_2$ as the CO_2 concentration in the mesophyll decreases. The delta ^{13}C values reflected the balance between enzymatic and diffusion processes in photosynthesis. Photosynthetic rate was significantly decreased by O_3 in older (25 d) but not in younger (19 d) fully expanded radish leaves, showing that the physiological age of the foliage at the time of measurement may be important. Some studies have shown that older leaves are more sensitive to O_3 than are younger leaves (e.g. Constantinidou & Kozlowski, 1979). In addition, younger radish leaves which matured

after O_3 exposures had begun may have compensated for the decreased supply of CO_2 available, whereas older leaves which matured prior to O_3 exposure may have lacked the capacity to adjust. Walmsley *et al.* (1980) found that radishes were capable of acclimating to continuous O_3; new leaves formed in O_3 were not as sensitive as foliage formed in O_3-free air. Determining whether the resistance of photosynthesis to O_3 in young radish leaves observed in the present study was due to acclimation or age effect was beyond the scope of this study.

The O_3-caused decrease in conductance, when photosynthesis was unchanged, resulted in an increase in WUE because the transpiration rate was decreased as a consequence of stomatal closure. At first glance this might appear to be beneficial to the plant, but because growth and root:shoot ratios were decreased by O_3, the consequences of O_3 damage are potentially severe in habitats where water and nutrients are limiting.

The changes in C_i and delta ^{13}C values resulting from O_3 treatments are in agreement with studies of plant responses to drought stress. Delta ^{13}C values were reported to increase with salinity in two halophytes (Guy, Reid & Krouse, 1980). In addition, *Cicer arientinum* plants exposed to drying cycles had lower C_i and greater delta ^{13}C values than did controls (Winter, 1981), and delta ^{13}C values of C_3 halophytes increased after three months of drought (Winter *et al.*, 1982). Farquhar and Richards (1984) found an increased WUE and a corresponding decrease in discrimination against ^{13}C with drought severity.

Freyer (1979) observed a 1.2‰ increase in delta ^{13}C values for sulphur dioxide (SO_2)-fumigated barley. This isotopic shift is similar in direction and magnitude to those reported here for O_3-fumigated plants, and it can be concluded that it resulted from SO_2-caused decreases in C_i and stomatal conductance. Other workers have observed that SO_2 can increase conductance (e.g. Biscoe, Unsworth & Pinckney, 1973) and decrease photosynthesis (Black & Unsworth, 1979). Such responses would result in an SO_2-caused increase in C_i and a decrease in delta ^{13}C value. This would be an isotopic shift

opposite in direction from those known for SO_2 and O_3. Thus stable carbon isotope analysis, coupled with gas exchange measurements, may help clarify the physiological effects of gaseous air pollutants on leaves. This approach is particularly important for pollutants such as SO_2 which can cause both increases and decreases in conductance. In addition, O_3 could conceivably cause increases in C_i, and concomittant decreases in delta ^{13}C values, if long-term exposures caused greater reductions in photosynthesis than observed in the present study. Such changes may occur for evergreen plants fumigated at chronic O_3 levels for long periods (months and years) or for plants predisposed to photosynthetic sensitivity to O_3 by biological and/or environmental factors.

The relationship between conductance and delta ^{13}C values of C_3 plant tissue may make it possible to readily evaluate the physiological impacts of stresses such as O_3 over time. A single measurement of delta ^{13}C values at the termination of an experiment may help interpret repeated measurements of conductance, C_i and photosynthesis taken over the course of air pollution studies. Future studies should focus on O_3-caused changes in delta ^{13}C values of carbon in several structural and non-structural pools and should evaluate stable isotopes of oxygen and hydrogen as further indicators of O_3 stress.

ACKNOWLEDGEMENTS

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Table IV.1. The effects of ozone (0.12 ul l^{-1}) on growth of radish (*Raphanus sativus*) and soybean (*Glycine max*) plants.*

	Leaf area (cm^2)	Below ground dry weight (g)	Above ground dry weight (g)	Root: shoot
Radish control	46.6 ± 1.3	0.065 ± 0.005	0.208 ± 0.010	0.313 ± 0.017
Radish O_3	41.0 ± 1.8	0.048 ± 0.006	0.176 ± 0.011	0.267 ± 0.016
Soybeanc ontrol	113.2 ± 1.4	0.272 ± 0.009	0.683 ± 0.011	0.398 ± 0.008
SoybeanO_3	102.4 ± 1.4	0.206 ± 0.009	0.603 ± 0.008	0.341 ± 0.013

* Means ± S.E.; all differences between control and O_3 within species are significant according to standard errors of the means.

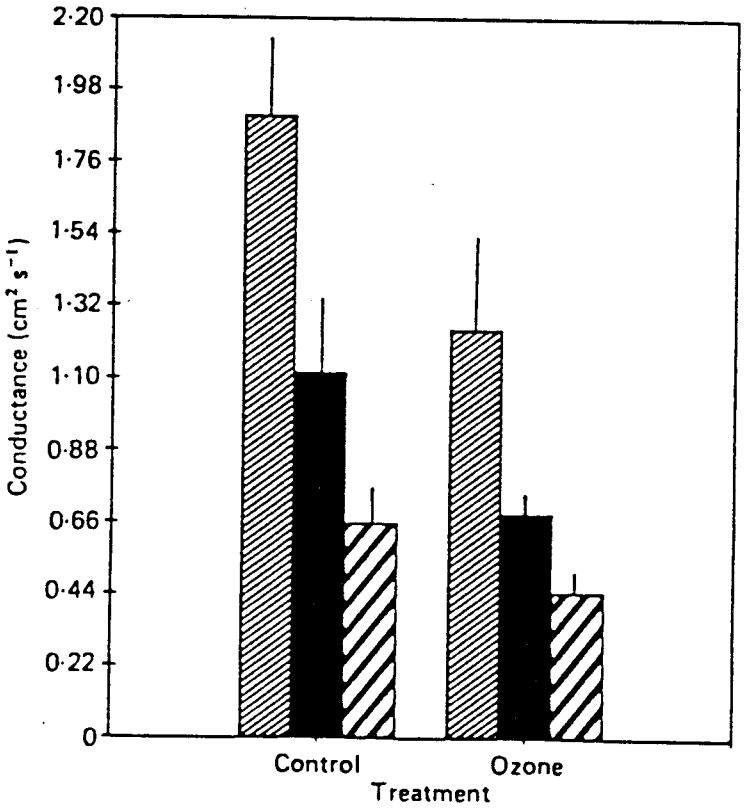

Figure IV.1. Conductance measurements (cm^2 s^{-1}) for leaves of radish (*Raphanus sativus*) and soybean (*Glycine max*) in O_3 (0.12 ul l^{-1}) or O_3-free air (control). Values are means (n=5 for 19 day control and O_3-treated and 25 day O_3-treated radish leaves; n=4 for control 25 day radish leaves; n=15 for soybean leaves) with error bars. Fine hatching: radish, 19 days old; black: radish, 25 days old; coarse hatching: soybean, trifoliolate.

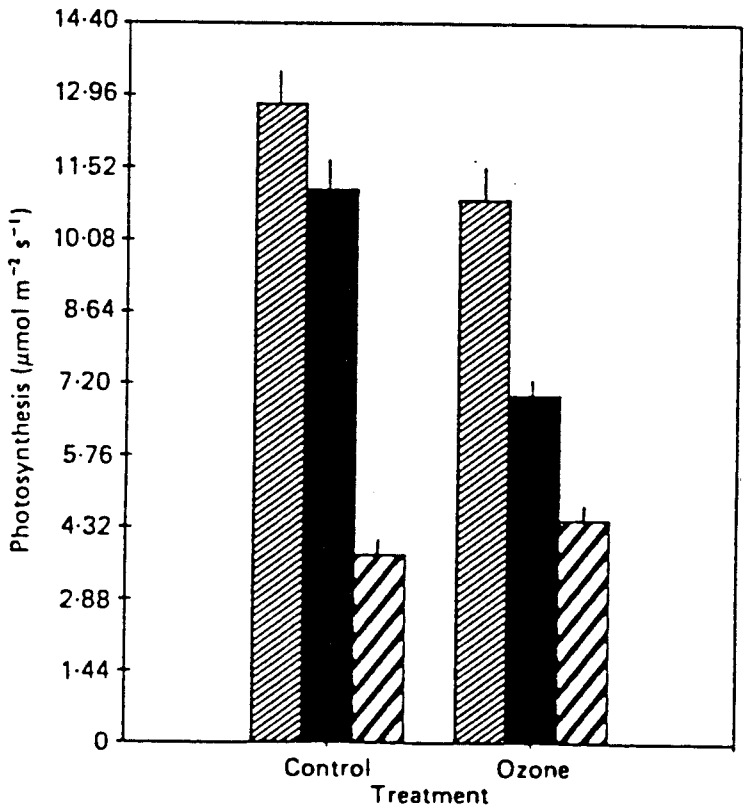

Figure IV.2. Photosynthesis measurements (umol m^{-2} s^{-1}) for leaves of radish (*Raphanus sativus*) and soybean (*Glycine max*) in O_3 (0.12 ul l^{-1}) or O_3-free air (control). Values are means (n=5 for 19 day control and O_3-treated and 25 day O_3-treated radish leaves; n=4 for control 25 day radish leaves; n=15 for soybean leaves) with error bars. For treatments see legend to Figure IV.1.

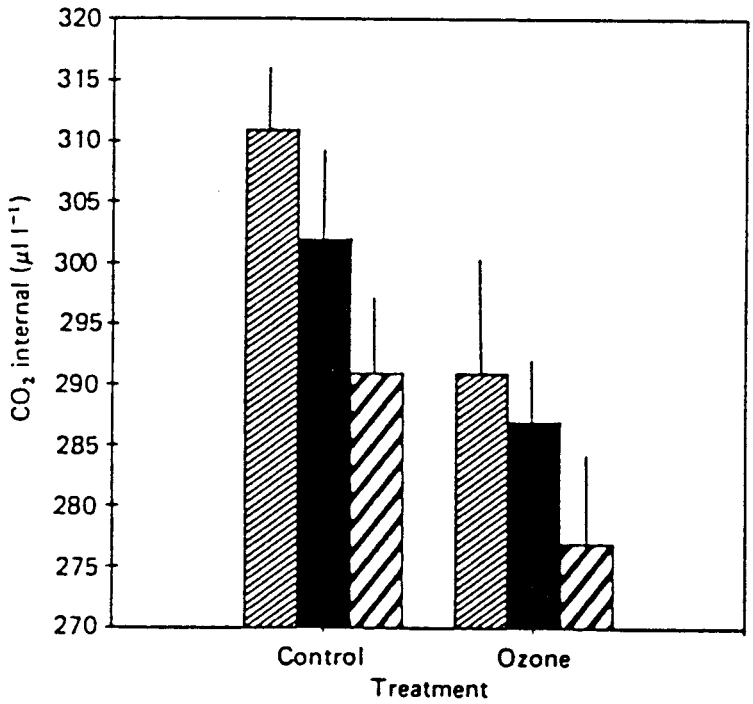

Figure IV.3. CO_2 internal concentrations (ul l^{-1}) for leaves of radish (*Raphanus sativus*) and soybean (*Glycine max*) plants in O_3 (0.12 ul l^{-1}) or O_3-free air (control). Values are means (n=5 for 19 day control and O_3-treated and 25 day O_3-treated radish leaves; n=4 for control 25 day radish leaves; n=15 for soybean leaves) with error bars. For treatments see legend to Figure IV.1.

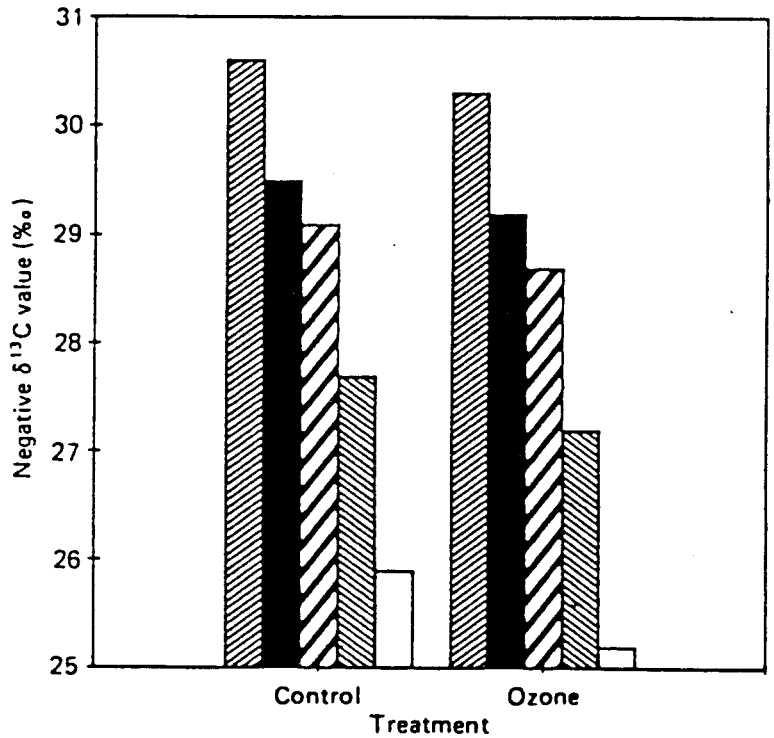

Figure IV.4. Negative delta ^{13}C values of radish (*Raphanus sativus*) and soybean (*Glycine max*) plant tissues exposed to O_3 (0.12 ul l^{-1}) and O_3-free air (control). Values are means (n=4) and standard errors are < 0.12. Standard is PDB. Fine hatching rising to the right: radish leaves; black: radish hypocotyls; coarse hatching: radish roots; fine hatching rising to the left: soybean leaves; white: soybean roots.

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V. RESPONSES OF ASPEN TO OZONE, NUTRIENT DEFICIENCY, AND DROUGHT: ANALYSIS OF WHOLE CANOPIES

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SUMMARY

Gas exchange and leaf area measurements were made on aspen (*Populus tremuloides* Michx.) foliage of different ages exposed to either charcoal-filtered air (CF) or ozone (O_3) in field chambers. Two experiments were conducted: one involving nitrogen (N) deficiency and the other, drought during the first 6 weeks of fumigation, with measurements taken after termination of the drought treatment. Total leaf area was reduced by all stress treatments. Net photosynthesis (*A*) declined with N deficiency within both air pollution treatments. Prior exposure to drought stress alone increased *A* for all but the oldest leaves, and *A* of middle-aged leaves of formerly droughted plants declined with O_3. *A* of older leaves decreased, and of younger leaves increased, in response to O_3 in both high and low N plants. Carboxylation capacity declined in older, and increased in younger, leaves of O_3-treated well-watered high N plants. Thus physiological maturity and senescence were accelerated by O_3. Whole plant carbon gain (WPCG) was reduced by O_3 and N deficiency alone and in combination via reductions in both total leaf area and whole plant photosynthetic rate (WPPR). WPCG declined in plants formerly exposed to drought due to declines in leaf area; in CF plants, enhanced WPPR partially compensated for these declines. Within the drought stress treatment, O_3 had little effect on leaf area but CG was reduced in some leaves due to declines in *A*. Analysis of whole canopies provided information about compensatory mechanisms impossible to obtain from more limited sampling.

Key Words: drought, nitrogen, ozone, photosynthesis, Populus

Abbreviations: *A* (net photosynthesis in umol m^{-2} s^{-1}), CF (charcoal-filtered air), CG (carbon gain for an individual leaf: net photosynthesis times leaf area, in umol s^{-1}), C_i (CO_2 concentration in the mesophyll, or CO_2 internal, in ul l^{-1}), g (stomatal conductance in mol m^{-2} s^{-1}), PAR (photosynthetically active

radiation, 400-700 nm, in umol m^{-2} s^{-1}), WPCG (estimate of whole plant carbon gain), WPPR (whole plant photosynthetic rate, WPCG divided by total leaf area, in umol m^{-2} s^{-1})

INTRODUCTION

Air pollutants such as ozone (O_3) that are distributed over vast regions are likely to impact plants already coping with natural stresses, even in remote locations. Ozone acting alone is known to suppress growth by affecting photosynthesis and other physiological processes, and the integrity and function of membranes. As with other environmental stresses, O_3 causes numerous changes in physiology and growth that taken together represent adjustments to environmental change. The extent to which plants can compensate to O_3 stress, that is, minimize growth reductions, is unknown. In addition, little is known about the potential for natural stresses to reduce the capacity of plants to compensate to O_3 stress.

O_3 stress

O_3 reduces photosynthesis (*A*) (reviewed by Darrall, 1989) and alters stomatal conductance (g) (reviewed by Winner *et al.*, 1988) in many plant species. Sensitivity of *A* to O_3 depends on leaf age, with younger leaves being more resistant than older leaves (Reich, 1983; Greitner & Winner, 1988). O_3 causes accelerated senescence of foliage (Reich, 1983; Amundson *et al.*, 1987). Evidence of foliar acclimation to O_3 absorption has been provided for radish (Walmsley, Ashmore & Bell, 1980). Stomata of leaves that expanded during O_3 fumigation were less sensitive than leaves of the same age transferred from CF air to O_3, and younger leaves developed more rapidly and emerged earlier in O_3. Increased sensitivity to O_3 with age may have two causes: an intrinsically greater sensitivity with physiological age, or the greater accumulated O_3 absorption due to longer exposure to O_3.

Stress combinations

Most work on nutrient stress-O_3 interactions has focused on visible foliar injury. Decreasing N levels is known to both increase (MacDowall, 1965) and decrease (Leone, Brennan & Daines, 1966) foliar injury from O_3 fumigations. Few reports are available on the effects of the combination of N deficiency and O_3 on plant physiology and growth. High N concentrations made *Lemna minor* more susceptible to loss of chlorophyll induced by O_3 (Craker, 1971). Biomass of radishes raised at optimal N was reduced by O_3, but effects of O_3 could not be detected in N deficient plants (Pell *et al.*, 1990). O_3 caused no change in *A* of alder with N_2-fixing symbionts, and a 40% decline in alder lacking symbionts, although the effect was not statistically significant (Greitner & Winner, 1989a). Thus improved N status due to symbionts increased resistance of *A* to O_3. O_3 and nutrient deficiency both reduced total leaf area in willow; leaf area of plants raised at the lowest nutrient level was more sensitive to O_3 than that of plants raised at the highest nutrient level (Greitner & Winner, 1989b). In general, increased N content of leaves results in increased g and *A* (Field & Mooney, 1986) and potentially greater pollutant uptake. How these changes in leaf physiology result in changes in O_3 impacts remains to be seen.

More is known about plant responses to the combination of drought and O_3. Drought generally lowers g, therefore decreasing pollutant uptake (Darrall, 1989). Lower g generally confers greater resistance to air pollutants (Winner & Mooney, 1980). Drought stress alone tended to decrease conductance but not photosynthesis in cotton, and severe drought stress reduced the negative effect of O_3 on carbon fixation (Temple *et al.* 1988). Drought protects plants from O_3 mainly through influence on stomatal aperture rather than through biochemical or anatomical changes (Tingey & Hogsett, 1985). However, information is lacking on gas exchange of foliage exposed to O_3 during recovery from drought.

Compensation to stress

Plants can compensate to stress by altering carbon allocation patterns. For example, O_3 increases the proportion of carbon allocated to shoots thereby decreasing the root:shoot ratio (Darrall, 1989); whereas N deficiency (Marschner, 1986) and drought (Sharp & Davies, 1989) have the opposite effect. Therefore, stresses may cause changes in the distribution of carbon partitioned to individual leaves, to whole canopies, and the entire plant. In radish, O_3 decreased *A* but increased efficiency of biomass accumulation relative to leaf area ratio; thus declines in relative growth rate were less than if the plant had not compensated by producing thinner leaves (Atkinson, Robe & Winner, 1988). The responses of plants exposed to two opposing stresses are difficult to predict, as the relative strength of the stresses in influencing allocation may vary with environmental conditions.

The WPCG rate of plants is a function of both whole plant leaf area and WPPR. Since plant stress responses include both changes in leaf area and changes in *A*, an understanding of the underlying mechanisms of stress response requires partitioning changes in WPCG between these two components.

Objectives

In this study, gas exchange and leaf area of aspen (*Populus tremuloides* Michx.) seedlings exposed to N deficiency, previous drought stress, and/or O_3 were measured. Experiments were designed to accomplish the following objectives:

1) Determine how leaf responses to stress differ with age;

2) Determine the mechanisms of changes in photosynthesis for leaves exposed to O_3 and other stresses;

3) Determine how plants minimize decreases in WPCG caused by stress by altering *A*, leaf number, and leaf area.

MATERIALS AND METHODS

Plant culture

The data reported here were acquired as part of a study of growth and physiology designed and conducted at the Pennsylvania State University (Pell, personal communication). Aspen, a deciduous woody perennial with leaves amenable to gas exchange measurements, was chosen for study because the effects of O_3 combined with other stresses are not as well known for trees as for annual crop species. Seeds were obtained from the US Environmental Protection Agency Research Laboratory in Corvallis, OR. In April 1990, seeds were germinated in a filtered-air greenhouse at Pennsylvania State University in peat containers filled with a 1:3 mixture of Metromix 500 and Redi Earth (W.R. Grace and Co., Cambridge MA). At age 5-6 weeks seedlings were transplanted into pots containing 6 liters of Metromix 500 with Osmocote and Micromax (Sierra Chemical Co., Milpitas CA) time-release fertilizers, gypsum (5.34 grams per pot), and aluminum sulfate (5.34 grams per pot). Plants were raised in 22 open-top field chambers with rain exclusion covers at the Rock Springs Research Farm near University Park, PA from June to September. Seedlings were supported with stakes and sprayed with Kelthane or Mavrik weekly to control mites. Lateral branches of trees were removed so that growth only occurred from the terminal bud, thus minimizing self-shading. This produced a growth form with a single stem so that the sequence of leaf formation and age was obvious.

Stress treatments

Aspen seedlings in the chambers received a series of environmental regimes involving O_3, drought, and N levels. Plants were exposed to either charcoal-filtered air (CF) or a simulated O_3 profile based on several years' monitoring data in Pennsylvania (Pell *et al.*, 1990). O_3 was administered in a diurnal pattern between 10:00 and 18:00 hours daily, with concentrations approximately ranging from 0.045 to 0.070 ppm. O_3 was generated

from O_2 and monitored by Thermo Environmental analyzers in a computer-controlled fumigation system. Exposures to O_3 began 5 days after plants were placed in chambers, on 11 June 1990.

Two experiments were conducted, one involving N stress and the other drought stress. Both experiments utilized the same "nonstressed" well-watered high N plants (WW,100%N) as the standard of comparison. In the first experiment, N level in the potting mix was varied, holding all other nutrients constant (Table V.1). "High N" plants received 100% of the level of time-release fertilizer recommended for aspen (Osmocote 14-14-14 NPK, 3.5 grams per pot) in one application at the time of transplantation. "Low N" plants (WW,25%N) received 25% of this amount supplemented with 0-38.3-0 calcium phosphate and 0-0-46.9 potassium sulfate to maintain the same concentrations of P and K as for the high N plants. The term "N deficient" is used to refer to low N plants in a relative sense in this study, as low N plants were lacking in N compared with high N plants.

In the second experiment, drought occurred during the first six weeks of exposure to O_3 in plants provided with 100%N (early drought, or ED,100%N) (Table V.1). Each treatment was replicated in four chambers. Moisture content of the potting medium was determined by time domain reflectometry (Drungil, Abt & Gish, 1989; Grantz, Perry & Meinzer, 1990). Moisture content in pots of well-watered seedlings was allowed to fall to 50% of container capacity before rewatering to container capacity. Moisture content in pots of early drought-stressed plants was allowed to fall to 25% of container capacity before rewatering to 50% of container capacity. Drought stress ended after six weeks exposure to O_3 or CF air, then seedlings received the same watering treatment as well-watered plants for the remaining six weeks of the experiment. Several new leaves expanded after termination of drought: thus there were two populations of leaves, one that expanded under drought and the other, under well-watered conditions.

Data analysis

Data were analyzed comparing stress treatments both with the nonstressed treatment and within stress treatments. Means and their standard errors were calculated for *A*, g, and area and plotted against leaf number (Sigmaplot, Jandel Scientific Corporation). Differences in *A*, area, and CG between treatments were detected with a non-parametric test (Statgraphics Mann-Whitney pairs test, Statistical Graphics Corporation) using a p-value of 0.05.

Gas exchange measurements

Gas exchange measurements were made with a LICOR LI-6200 Portable Photosynthesis System (LI-COR Inc., Lincoln NE) equipped with a 250 ml cuvette. The measurement period commenced 52 days after the fumigation began and continued for three weeks. Plants in the early drought treatment had over one week of well-watered conditions when gas exchange measurements began; thus foliar recovery from drought was studied. Before daily gas exchange measurements, both well-watered and early drought-stressed plants were watered to container capacity. The first four leaves below the seedling apex were too small and fragile to measure. The fifth leaf from the apex of a randomly selected plant was tagged as leaf #1 (first measurable leaf) each day and leaves were numbered and measured down the stem. Thus over the three week measurement period, leaves given the same number were in a comparable developmental condition. For the first three days, gas exchange was measured on every leaf of a total of eight plants, at least one from each of the six treatments. Because of the similarity in values between adjacent leaves, on subsequent days every other leaf (odd numbered leaves) was measured in order to sample a larger number of plants. From two to six plants were measured per day, with O_3 and CF plants within the other treatments measured on the same day. Measurements were alternated so that the same treatment was not always sampled at the same time of day.

Measurements of O_3-treated plants were taken during fumigation with O_3. Ambient CO_2 concentrations were less than 340 ul l^{-1} at

onset of measurements, and a quartz halogen lamp illuminated the leaf when ambient PAR fell below 1000 umol m^{-2} s^{-1}. Photosynthesis values reported here are net photosynthesis (gross photosynthesis minus respiration); these experiments were not designed to distinguish stress effects on gross photosynthesis and respiration. Ten to 14 plants per treatment were measured.

CO_2 response (A/C_i) curves

The LICOR LI-6200 System was used to generate A/C_i curves (D. McDermitt, LICOR, personal communication) on 25-29 August. A leaf was enclosed in the cuvette at ambient [CO_2] and allowed to deplete [CO_2] to the compensation point. At this point, high [CO_2] was introduced by breathing into the cuvette to bring C_i over 800 ul l^{-1}. The leaf was allowed to draw [CO_2] down until C_i values overlapped those obtained initially at ambient [CO_2]. Curves were generated for a young leaf (number 5) and older leaf (31 for the first curve, 25 for the next nine) of ten each well-watered, high N plants exposed to CF air or O_3 over five days. Initial slopes of the linear portions of the curves (from ambient [CO_2] to the compensation point) and the CO_2 compensation points were calculated.

Leaf area

Leaf area was determined nondestructively for every leaf of each plant sampled for gas exchange using a LICOR LI-3000A area meter. Values reported are for one side of a leaf. Leaf areas were summed for each plant and means for each treatment were calculated. The number of leaf scars per plant was counted and expressed as a percent of the total number of leaves produced (leaves plus leaf scars) to quantify leaf abscission.

Whole plant carbon gain

Carbon gain (CG) was calculated by multiplying *A* times area for each measured leaf. The first eight plants had *A* and area measurements for all leaves and therefore WPCG for the whole plant could be calculated directly by summing the values for each leaf.

Whole plant carbon gain (WPCG) for the remainder of the plants in which *A* values were only available for every other leaf was estimated. *A* for unmeasured leaves was interpolated as the average of the *A* values of the two neighboring measured leaves. Thus *A* for leaf 6 was estimated as the average of *A* for leaves 5 and 7. The estimated *A* value was multiplied by the measured area value and products for individual leaves (both those with measured and estimated values for *A*) summed to estimate WPCG for each plant. Data from the eight plants in which every leaf was measured for *A* were used to test the accuracy of this method. Estimates for WPCG were within 3% of those obtained directly from measurements for these eight plants (data not shown). Values for WPCG were divided by total plant leaf area to calculate whole plant photosynthetic rate (WPPR).

RESULTS

Leaf area and number

The area and number of leaves are known to change in response to stress. Leaf number reflects both the formation of new leaves and the abscission of old leaves. Leaf abscission was increased by O_3 alone and in combination with N deficiency, but by no other treatments (Table V.2). Thus O_3 reduced leaf number in well-watered plants at both N levels by accelerating senescence of older leaves. In contrast, early drought decreased leaf number by reducing the total number of leaves formed. Abscission was unaffected by early drought either alone or combined with O_3.

Total plant leaf area was reduced by all stress treatments (Table V.2). Within the low N treatment, total leaf areas of O_3 and CF plants were not significantly different. Within the early drought, high N treatment, there was no effect of O_3 on whole plant leaf area.

Photosynthesis

Photosynthesis decreased in older leaves, but increased in younger leaves of well-watered, high N plants exposed to O_3 (Fig. V.1a, Table V.3). Younger leaves therefore partially compensated for the diminished photosynthetic capacity of older leaves, and the profile of change in *A* with age was altered by O_3. In contrast, Reich (1983) reported that O_3 accelerated the decline in *A* with age in hybrid poplar leaves, but patterns of change were similar in O_3-treated and CF plants. The differences between the two studies could be attributed to species, O_3 concentration, or light levels. Perhaps compensation could not occur at the higher O_3 concentration used by Reich (1983).

N deficiency tended to reduce *A* of mature and older mature leaves of well-watered plants in CF air (Figs. V.1a & b). Statistically significant differences were detected for leaves 11,13,19,21,25, and 29. Prior exposure to drought enhanced *A* in young and mature leaves (Figs. V.1a & c). The differences were statistically significant for leaves 5,11,15,17, and 19. The mature leaves were formed during drought, whereas young leaves expanded after termination of drought.

The patterns of change in *A* with leaf age and O_3 within the well-watered, low N treatment were similar to those of the well-watered high N plants (Figs. V.1a & b) but the means were consistently lower in the low N treatments. The differences in *A* between CF and O_3-treated young leaves were less often statistically significant in the low N than the high N treatments (Table V.3). Thus ability of *A* of younger leaves to compensate for *A* of older leaves depended upon N supply.

Effects of O_3 on *A* within the early drought, high N treatment were not as great as for the other treatments. *A* of middle-aged leaves only was depressed by O_3 (but the effect was not statistically significant, Table V.3). The profile of *A* across leaf age of early drought, high N, O_3 plants was more similar to all the CF treatments than to the other O_3 treatments. Presumably during the drought period, foliage had taken up less O_3 as a result of

lower g than in well-watered foliage. Gas exchange measurements of leaves of other aspen plants during exposure to drought showed that the drought conditions applied in this study were severe enough to lower *A* and g (data not shown). Within the O_3 treatments, N deficiency caused declines in *A* for all but the youngest leaves (Fig. V.1a & b), and early drought treatment increased *A* of middle-aged leaves (Fig. V.1a & c).

Stomatal conductance

Stomatal conductance was measured because changes in g can affect both *A* and O_3 absorption rates. For all treatments, changes in g with leaf age generally followed the same patterns as for *A*, although g of the youngest leaves was not affected by any treatment (Fig. V.2a, b & c). Conductance of middle-aged leaves of well-watered high and low N plants was significantly reduced by O_3. N deficiency alone had little effect on g, but the combination of N deficiency and O_3 caused declines in g of all but the youngest leaves. The highest conductance values were measured in early drought, high N, CF leaves. Within the early drought treatment, g of all but the youngest leaves tended to decrease with O_3.

Mechanisms of changes in photosynthesis caused by O_3

Changes in g due to O_3 were less pronounced than changes in *A* in most treatments, suggesting that there were direct effects of O_3 on photosynthesis. Photosynthesis was plotted against C_i in order to evaluate the effects of stresses on carboxylation capacity and capacity to regenerate ribulose bisphosphate (RuBP) (von Caemmerer & Farquhar, 1981). Typical A/C_i curves of two ages of leaves of well-watered high N plants are shown in Fig. V.3.

The initial slope, and therefore carboxylation capacity, was decreased in older leaves but increased in younger leaves by O_3 (Table V.4). The CO_2 saturation point was depressed in O_3-treated older leaves (Fig. V.3); thus RuBP regeneration capacity was diminished. Similar reductions in carboxylation efficiency and RuBP regeneration capacity have been reported in O_3-treated radish leaves

(Atkinson, Robe & Winner, 1988). The CO_2 compensation point in older aspen leaves increased with O_3, but the inverse was true in young leaves (Table V.4). This suggests that O_3 enhanced respiration in older leaves. O_3 caused accelerated maturation of young leaves as well as senescence of older foliage.

Leaf area

Leaf area increased with age until it peaked in middle-aged leaves, then declined with age in WW CF plants of both N treatments (Fig. V.4a & b). N deficiency reduced area of the middle-aged leaves within both the well-watered CF and O_3 treatments; the differences were statistically significant for leaves 5-15 and 21-29. Leaf area of younger O_3-treated leaves was increased, and of middle-aged leaves decreased, in the continuously well-watered plants. The effect was more pronounced in high N plants. Abscission of older foliage of O_3-exposed well-watered plants may have allowed mobilization of N from older to younger leaves, permitting compensatory increases in leaf area and *A* in younger foliage. Due to the high demand for N in photosynthetic enzymes and reactants, *A* is positively correlated with foliar N content (Field & Mooney, 1986).

Leaf area within the early drought treatment was unaffected by O_3 (Fig. V.4c). During drought, aspen seedlings produced smaller (statistically significant for leaves 13-33) and fewer leaves, which could explain the enhanced rates of *A* in this treatment. Plants exposed to early drought were likely to have had high foliar N levels, as 100% N was available to plants which were much smaller than in the continuously well-watered high N treatment. The area of the leaves formed after termination of drought was larger than that of corresponding well-watered high N plants for both the CF and O_3 treatments (Fig. V.4a & c). The differences were statistically significant for leaves 3-7. This enhanced growth was likely a consequence of a flush of N available at the onset of well-watered conditions for construction of larger leaves. Apparently foliar N concentrations were sufficient even in these larger leaves to permit

an increase in *A* over that in continuously well-watered plants.

Whole plant carbon gain and photosynthetic rate

WPCG was calculated as the sum of the product of photosynthesis rates and areas of individual leaves. All stress treatments, single and multiple, reduced WPCG compared with unstressed plants (Fig. V.5a, b & c; Table V.5). For continuously well-watered plants, the percent change in leaf area with stress was similar in magnitude to that of WPPR; thus alterations in *A* and area due to stress contributed about equally to changes in WPCG.

Plants subjected to early drought (ED,100% N) had a different response (Fig. V.5c). Changes in WPCG within this treatment due to O_3 were of lesser magnitude than for the other treatments and were attributable only to changes in WPPR, as O_3 did not alter area (Table V.5). The decline in leaf area with early drought alone was partially offset by an enhancement of WPPR following termination of drought, therefore the impact on WPCG was reduced. The entire reduction in WPCG in the early drought, high N, O_3 treatment was attributable to reduced leaf area, as there was no compensatory increase in WPPR.

DISCUSSION

Modification of stress response with leaf age

1). Old leaf responses and accelerated senescence

Aspen seedlings in this study exhibited two components of senescence: declines in *A* with age and abscission of the oldest leaves. The three types of stresses appeared to have different effects on senescence. N deficiency had no effect on either component of senescence, O_3 accelerated both components of senescence, and drought stress delayed senescence.

Accelerated senescence caused by O_3 has conventionally been regarded as detrimental, or as damage. However, the results of these experiments suggest that accelerated senescence may be

adaptive. Leaves with diminished rates of *A* were shed, and nutrients and other resources made available for export to more productive foliage. Thus this study shows that younger leaves may be able to grow larger and have higher *A* rates in O_3 than in CF air. Although these compensatory changes may not be sufficient to completely offset the effects of O_3 stress, the impact of O_3 is less severe then would be expected in the absence of such changes. Even a slight degree of compensation might be sufficient to ensure plant survival during long-term exposure to O_3. N level appears to be crucial in compensation.

2). Stress resistance of young leaves

In this study, young leaves were more resistant to O_3 than were old leaves. Whether the increase in sensitivity to O_3 with age was due to physiological changes associated with aging, or to the increase in accumulated dose of O_3 with age, or both, could not be concluded from these data.

Mechanisms of changes in photosynthesis rate caused by stress

1). Partitioning decreases in *A* between mesophyll and stomatal factors

Decreases in *A* resulting from stresses may be due to stomatal closure and/or altered metabolic capacity in the mesophyll. It is essential to identify the relative importance of these two mechanisms, in order to assess repair costs and long-term changes in plant function that may occur as consequences of stress. Leaves can respond to O_3 and other air pollutants by closing stomata, thereby excluding the pollutant from the mesophyll and preventing damage to cells. Exclusion of pollutant due to stomatal closure occurs at the cost of decreased potential carbon gain. Additional consequences of stomatal closure include a potential increase in leaf temperature and conservation of water.

If stomata do not close enough to exclude O_3, damage to components of the photosynthetic apparatus can occur. Photosynthesis of aspen seedlings in this study responded to O_3 with partial stomatal closure. In addition, the non-stomatal components

of *A* were also affected, at least in the well-watered high N treatment.

2). Changes in carboxylation capacity and RuBP regeneration

Analysis of A/C_i curves showed both carboxylation efficiency (RuBisco activity) and RuBP regeneration (and thus electron transport capacity) were impaired in older leaves by O_3. Such direct injury to the photosynthetic apparatus could increase the demand for energy, carbon compounds, and mineral nutrients for repair. There could be substantial repair costs in leaves chronically exposed to O_3, at the same time that carbon fixation was impaired due to decreased efficiency of photosynthesis.

Similar biochemical changes result from normal processes associated with senescence in non-stressed vegetation. It is tempting to consider changes in leaf metabolism caused by O_3 to be identical with the changes due to natural senescence, but simply occurring earlier and faster than normal. Determining ways that the process of accelerated senescence caused by O_3 differs from natural senescence will be an important area for future research.

Mechanisms of changes in WPCG caused by stress

Changes in *A*, leaf number, and leaf area

Plants can potentially respond to stress by altering *A*, leaf area, and leaf number. The three stresses examined here altered these factors to different degrees. All three factors were affected by O_3, and the effect on leaf number was via abscission, not reduction in number of leaves produced. N deficiency had no effect on leaf number but decreased area and *A*. Drought decreased number and area of leaves produced; during recovery from drought *A* and area increased.

The timing of the stress in the plant's development is crucial in determining the response. For example, abcission of older leaves was severe in aspen which were well-watered during the first half of the fumigation period and drought stressed during the last half (Pell, unpublished data). In this case, abscission reduced transpiring leaf area and was an adaptive response to decreased

water availability. Thus, with drought stress late in development, as with O_3 stress, younger foliage is more resistant than older foliage to the stress in the sense that the oldest leaves are the first to be lost to compensate for the stress. With natural stresses occurring early in life, plants may adjust leaf area by limiting growth, but if the stress occurs later in development, abscission of older foliage may be the only possible compensatory response.

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Table V.1. Treatments in field chambers and experimental design for aspen (*Populus tremuloides* Michx.) exposed to various stress treatments.

TREATMENT CODE	CONDITIONS	# OF CHAM-BERS	# OF SAM-PLES
WW,100%N,CF	Continously well-watered, 100% N, CF air	4	14
WW,100%N,O_3	Continously well-watered, 100% N, O_3	4	14
WW,25%N,CF	Continously well-watered, 25% N, CF air	3	11
WW,25%N,O_3	Continously well-watered, 25% N, O_3	3	10
ED,100%N,CF	Early drought, 100% N, CF air	4	10
ED,100%N,O_3	Early drought, 100% N, O_3	4	10

Table V.2. Number of abscised leaves expressed as percent of the original number of leaves produced (AB), and total plant leaf area (LA) in cm^2 of aspen (*Populus tremuloides* Michx.) exposed to various stress treatments. N is number of plants measured; SE is standard error. Values within a column followed by different letters are significantly different at the 0.05 level (Mann-Whitney pairs test). See Table V.1 for treatment codes.

TREAT-MENT	N	AB MEAN	AB SE	AB	LA MEAN	LA SE	LA
WW,100%NCF	14	14.8	1.0	a	3181	112	a
WW,100%NO_3	14	23.7	2.0	b	2746	133	b
WW,25%N CF	11	13.3	1.9	a	2439	104	bc
WW,25%N O_3	10	23.6	2.2	b	2120	165	c
ED,100%NCF	10	13.8	1.8	a	1575	111	d
ED,100%NO_3	10	15.9	1.0	a	1471	140	d

Table V.3. Aspen (*Populus tremuloides* Michx.) leaves showing significant differences (Mann-Whitney pairs test, p=0.05) between CF and O_3 for photosynthesis (*A*), leaf area (LA, cm^2), and carbon gain (CG). Numbers are leaf numbers, from youngest to oldest.

TREAT-MENT	*A* O_3>CF	*A* O_3<CF	LA O_3>CF	LA O_3<CF	CG O_3>CF	CG O_3<CF
WW, 100%N	3-7	19-35	3-9	19-35	3-9	19-35
WW, 25%N		19-39		27		19-35
ED, 100%N			33	21	33	13-21

Table V.4. Initial slopes of A/C_i curves and CO_2 compensation points (C.P., in ul l^{-1}) for young leaves (number 5) and older leaves (number 25 or 31) of aspen (*Populus tremuloides* Michx.) exposed to CF air or O_3. N=10 for each treatment. Values in a column followed by the same letter are not significantly different (Mann-Whitney pairs test, p=0.05).

TREATMENT	MEAN of SLOPE	SE of SLOPE		MEAN of C.P.	SE of C.P.	
WW,100%N,CF young	0.0995	0.0095	a	79.9	3.7	a
WW,100%N,CF older	0.1083	0.0055	a	67.8	2.5	a
WW,100%N,O_3 young	0.1159	0.0053	a	70.2	3.4	a
WW,100%N,O_3 older	0.0517	0.0067	b	109.5	17.2	b

Table V.5. Estimates of whole plant carbon gain (WPCG, in umol CO_2 s^{-1}), whole plant photosynthetic rate (WPPR, in umol CO_2 m^{-2} s^{-1}), and changes in WPCG, leaf area (LA), and WPPR expressed as percent change from the WW,100%N,CF treatment, in aspen (*Populus tremuloides* Michx.) seedlings exposed to various stress treatments.

TREAT-MENT	WPCG, MEAN	WPCG,% CHANGE	LA, % CHANGE	WPPR, MEAN	WPPR, % CHANGE
WW 100%N CF	4.506	0	0	14.17	0
WW 100%N O_3	3.205	-28.9	-13.7	11.67	-17.6
WW 25%N CF	2.697	-40.1	-23.3	11.06	-21.9
WW 25%N O_3	1.744	-61.3	-33.4	8.23	-41.9
ED 100%N CF	2.594	-42.4	-50.5	16.47	+16.2
ED 100%N O_3	2.077	-53.9	-53.8	14.12	- 0.3

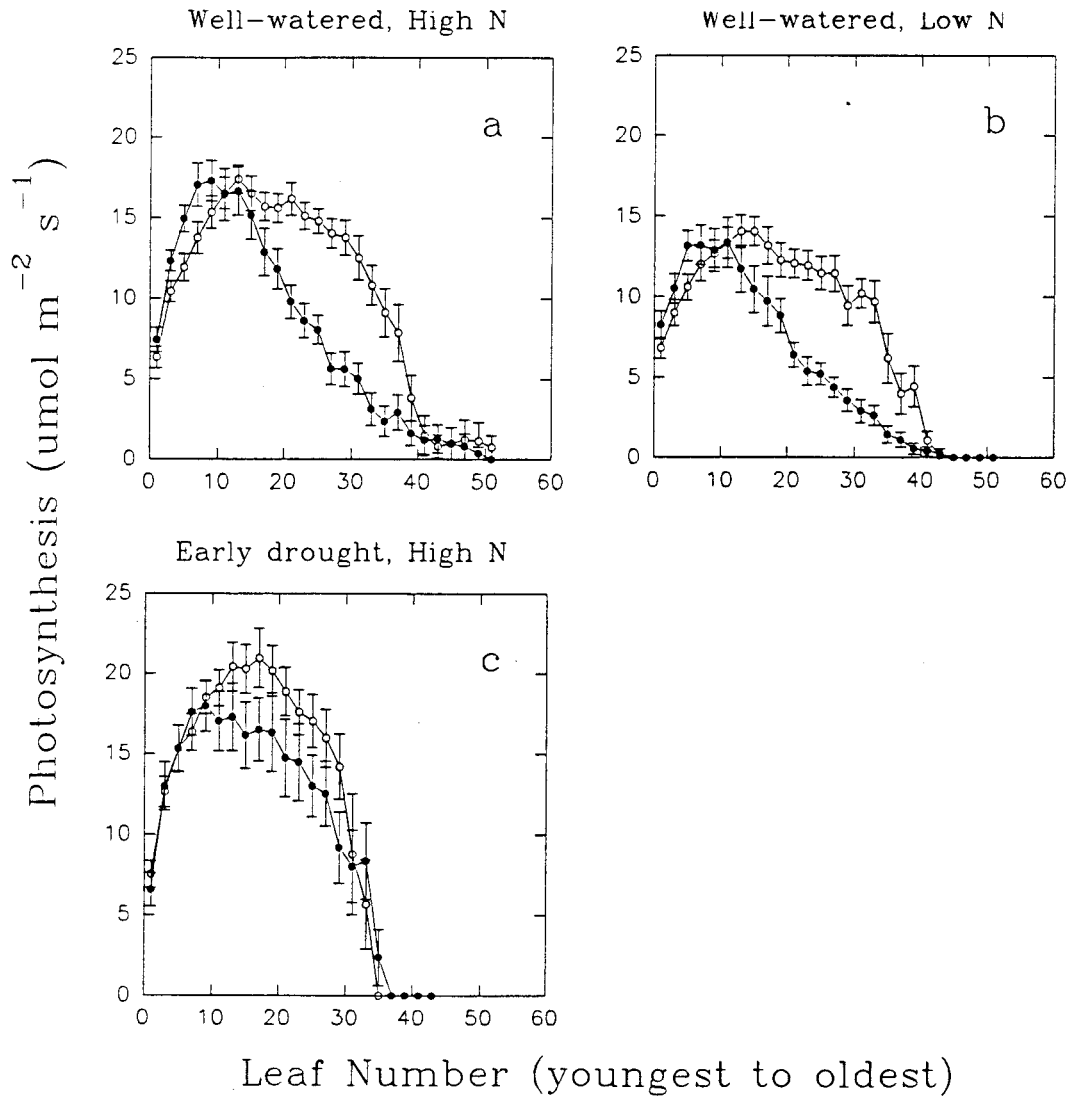

Figure V.1. Net photosynthetic rates *vs* leaf age for aspen seedlings exposed to various stress treatments. Symbols are means of 10-14 plants, with error bars. Open circles represent CF; filled circles represent O_3.

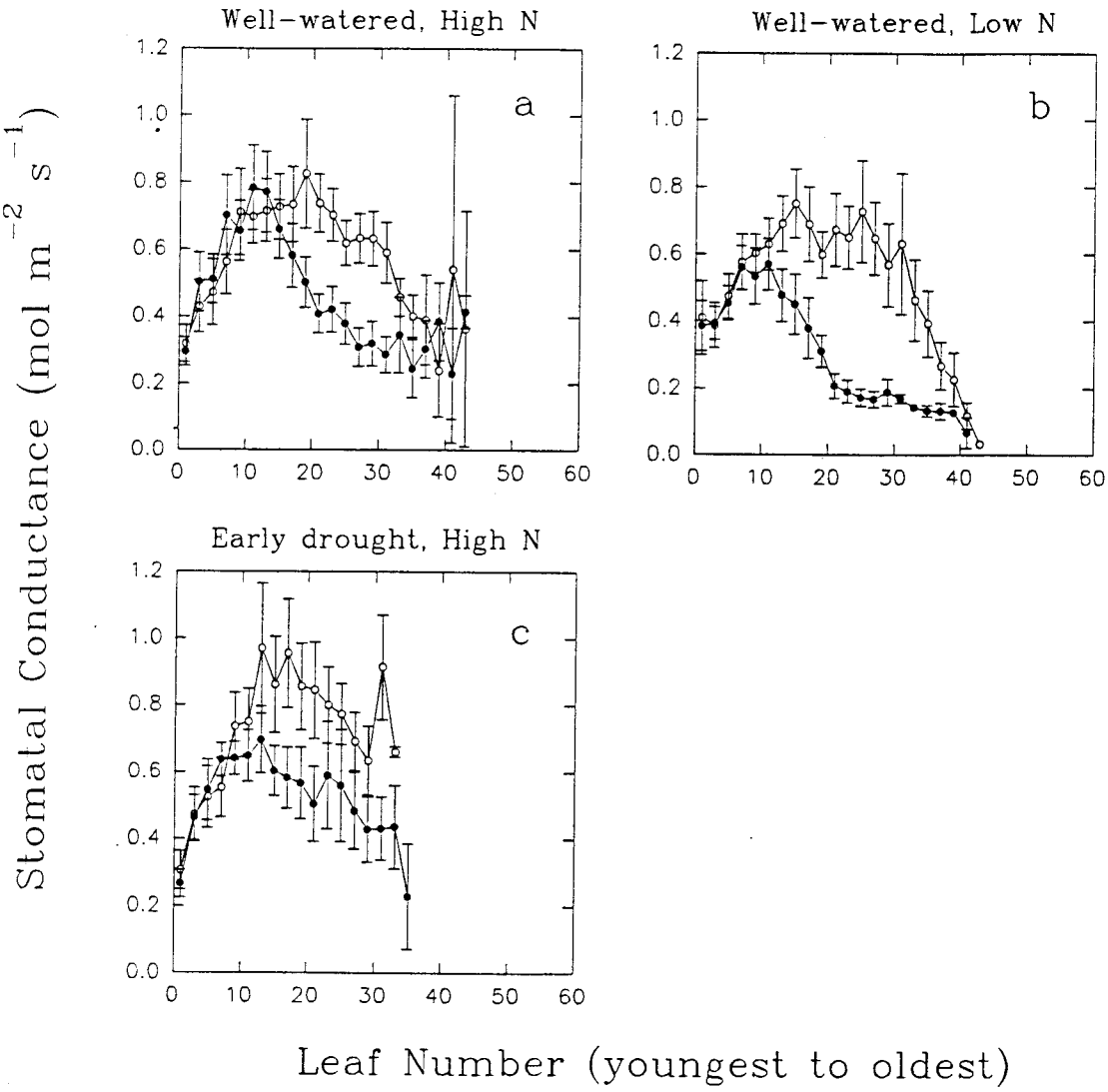

Figure V.2. Stomatal conductance *vs* leaf age for aspen seedlings exposed to various stress treatments. Symbols are means of 10-14 plants, with error bars. Open circles represent CF air; filled circles represent O_3.

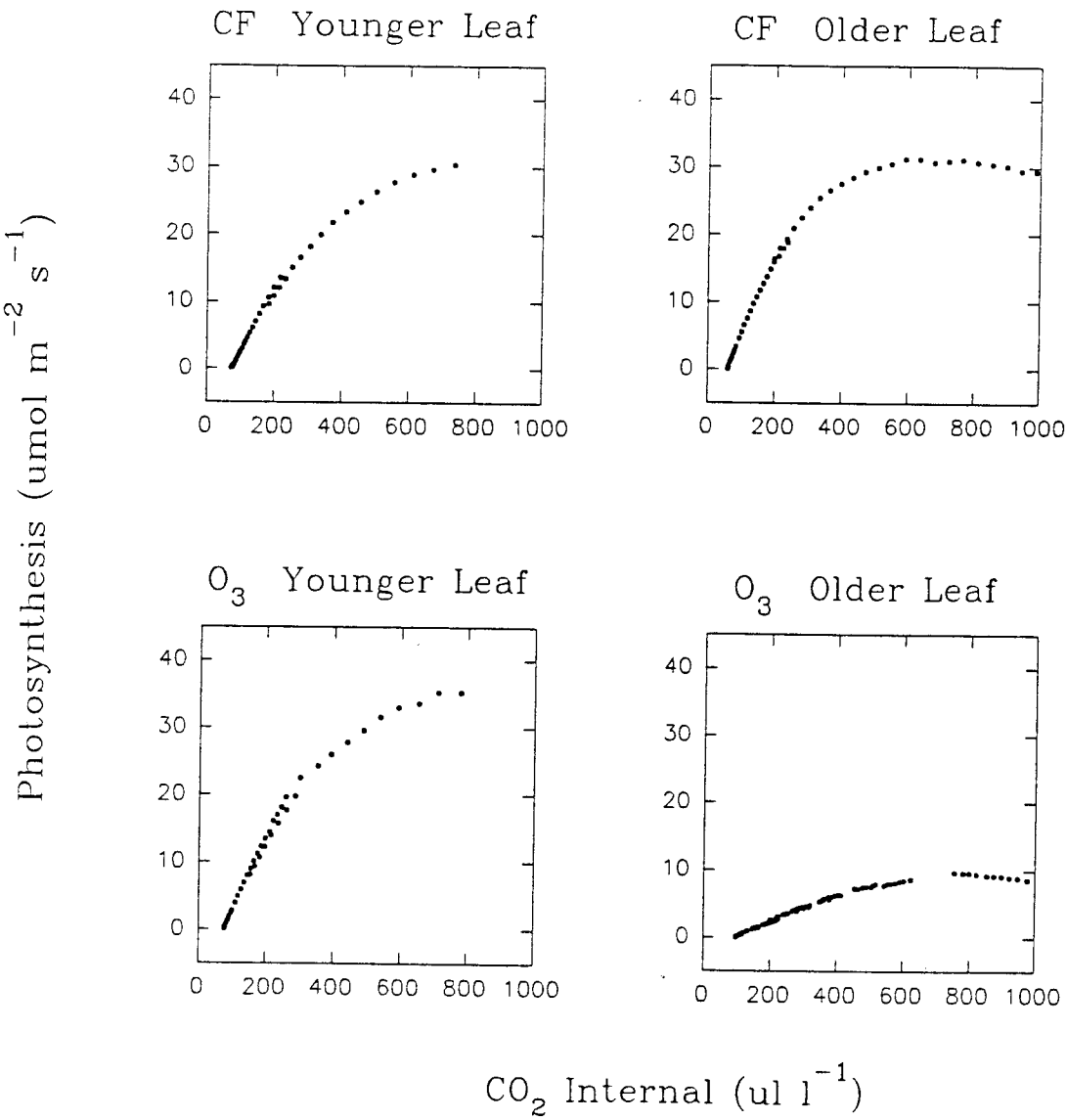

Figure V.3. Typical A/C_i curves for aspen leaves of two ages from the well-watered, 100% N treatment, exposed to CF air or O_3.

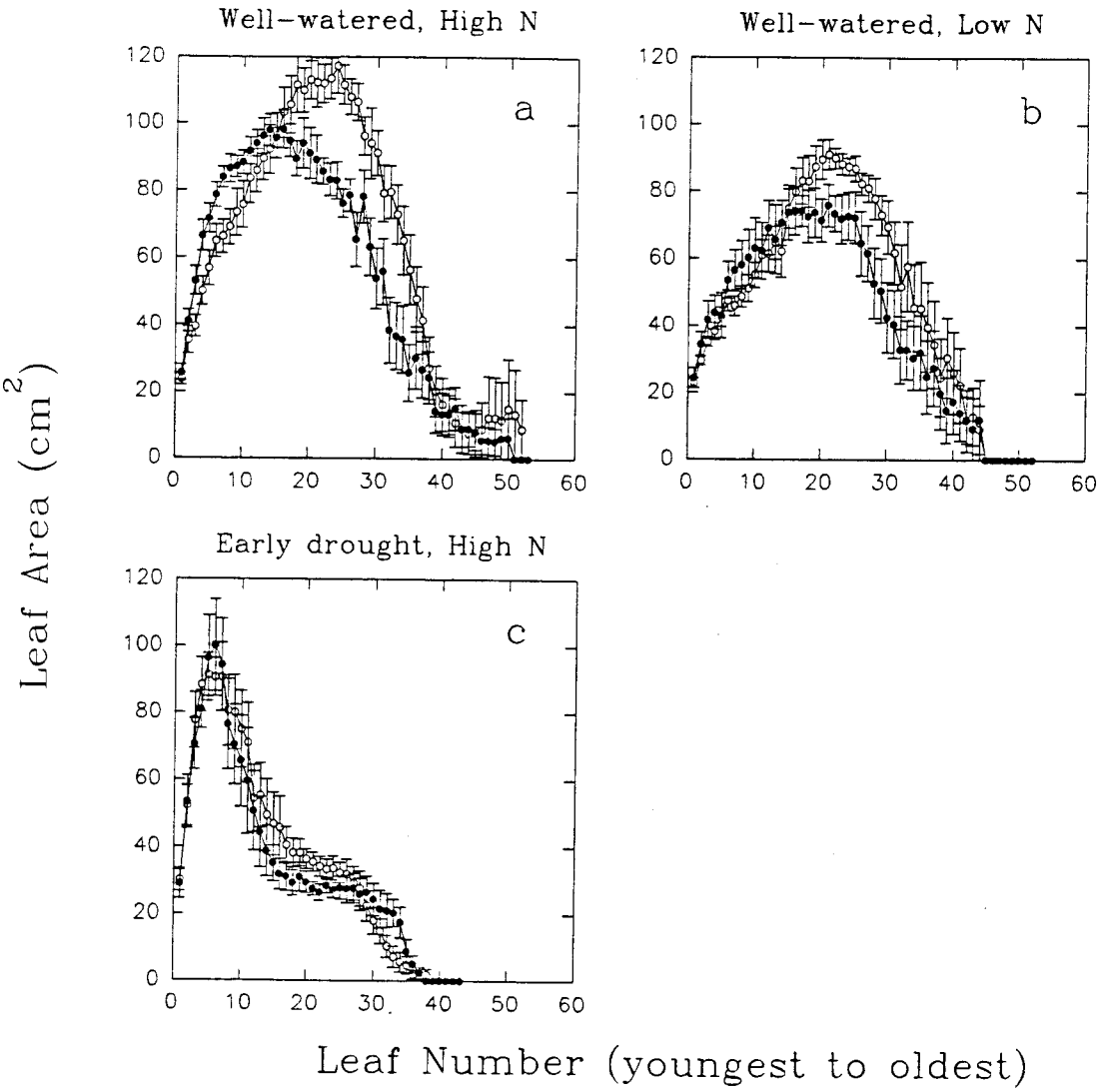

Figure V.4. Leaf area (cm^2) *vs* leaf age for aspen seedlings exposed to various stress treatments. Symbols are means of 10-14 plants, with error bars. Open circles represent CF; filled circles represent O_3.

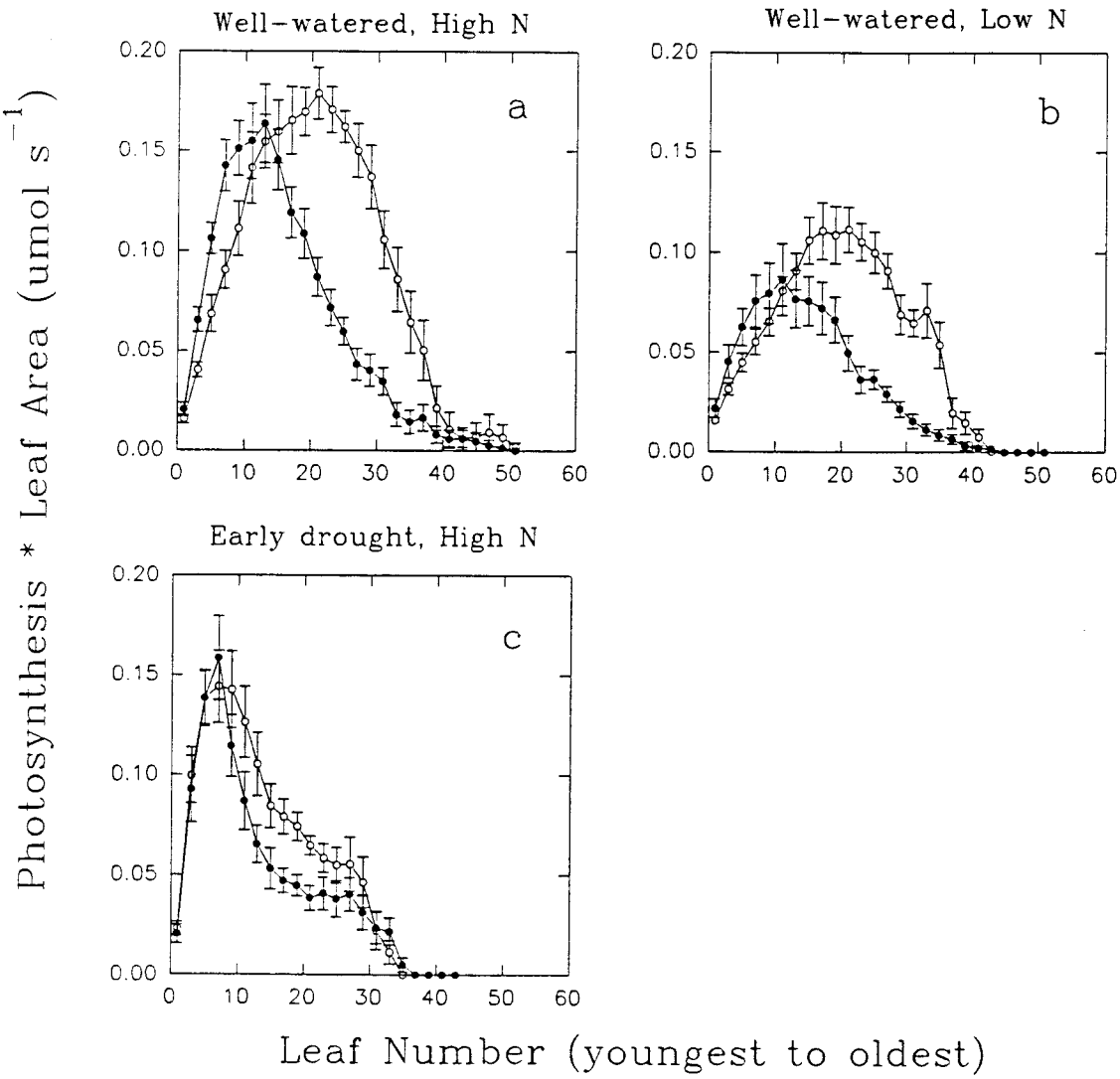

Figure V.5. Carbon gain (net photosynthesis multiplied by leaf area) *vs* leaf age for aspen seedlings exposed to various stress treatments. Symbols are means of 10-14 plants, with error bars. Open circles represent CF; filled circles represent O_3.

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VI. CONCLUSIONS

The research questions posed in the introduction to this thesis were addressed in the manuscripts. The questions are restated here, with brief conclusions drawn from each set of experiments.

1). How does nutrient level affect O_3-caused visible injury and growth?

a). Nutrient status affected response to O_3. High nutrient willows showed more visible injury, but low nutrient plants sustained the greatest percent reduction in leaf area caused by O_3.

b). Visible injury did not correspond with growth alterations.

c). Nutrient deficiency had greater impact than O_3 on resource allocation at the levels applied in this study.

d). Nutrient deficiency enhanced O_3-caused reductions in leaf area.

2). Can changes in shoot physiology with O_3 affect rhizosphere symbioses and therefore the ability of plants to acquire nutrients?

a). O_3 altered the relationship between photosynthesis and conductance. Recovery from O_3 effects was impeded in non-symbiotic alder relative to alder with rhizosphere symbionts.

b). Photosynthesis of leaves having the highest conductance was decreased by O_3.

c). O_3 disrupted host root cell integrity. Host root cells were more sensitive to O_3 effects than were *Frankia* cells.

3). Are photosynthesis measurements taken during a brief window of time representative of plant physiology throughout a prolonged exposure to stress?

a). Conductance and CO_2 internal decreased, and delta ^{13}C values of foliage and below-ground tissues increased with O_3.

b). Stable carbon isotope ratios can be used to integrate effects of O_3 on gas exchange over time.

c). Biomass and root:shoot ratios were reduced by O_3 in soybean and radish.

4). How are photosynthesis and leaf area of whole plants affected by O_3 in nitrogen deficient plants and in plants recovering from drought? Does the response vary with leaf age, and how do plants compensate to these stresses?

a). Photosynthesis and leaf area in young leaves of well-watered aspen increased with O_3, compensating for declines in older leaves. These responses were less pronounced in nitrogen-deficient seedlings.

b). Declines in photosynthesis with O_3 were associated with both stomatal and mesophyll factors.

c). Carboxylation efficiency decreased in older, but increased in younger leaves with O_3. RuBP regeneration capacity declined in older leaves with O_3.

d). Drought reduced leaf area; termination of drought increased photosynthesis and leaf area. Prior exposure to drought reduced the effects of O_3 on photosynthesis and leaf area.

e). Changes in whole plant carbon gain caused by O_3 could be partitioned about equally between changes in photosynthesis and leaf area in well-watered aspen, but to photosynthesis alone in droughted plants.

f). The different responses to the various stresses were all likely mediated through effects on foliar nitrogen level.

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